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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Neuronal and astrocyte cell cultures from the cerebellum and fibroblasts and epithelial cells from the skin and kidney (respectively) of DNA repair mutant mice were examined for the acute and delayed toxicity to nitrogen mustard (HN2) or the related alkylating agent methylazoxymethanol (MAM). Cerebellar neurons from DNA repair mutant mice (i.e., $XPA^{-/-}$, $MGMT^{-/-}$) were more sensitive to HN2 and MAM than comparably treated wild type or $AAG^{-/-}$ neurons. Cerebellar neurons from MGMT+ mice were protected from the acute toxicity of both MAM and HN2. A similar pattern of sensitivity was observed for long-term HN2- and MAM-treated cerebellar neurons or fibroblasts and epithelial cell lines from DNA repair deficient (i.e., $MGMT^{-/-}$, $AAG^{-/-}$) mice. In comparison to wild type and DNA mutant (i.e., $MGMT^{+}$, $AAG^{-/-}$) mice, the loss of cerebellar neurons, degeneration and altered dopaminergic neurons were especially evident in the brains of $MGMT^{-/-}$ mice administered MAM. These findings are consistent with HN2 and MAM selectively targeting neural and non-neural cells *in vivo* via a mechanism involving DNA damage. *In vivo* studies are currently underway with DNA repair-deficient mice to further examine the relationship between DNA damage and *in vivo* neurotoxicity of MAM and HN2.

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INTRODUCTION

Experiments are proposed to examine the molecular mechanism by which mustard chemical warfare agents induce neuronal cell death. DNA damage is the proposed underlying mechanism of mustard-induced neuronal cell death. We propose a novel research strategy to test this hypothesis by using mice with perturbed DNA repair to explore the relationship between mustard-induced DNA damage and neuronal cell death. Initial *in vitro* studies (Years 1, 2 & 3) are proposed to examine the cytotoxic and DNA damaging properties of the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2) and the neurotoxic DNA-damaging agent methylazoxymethanol (MAM) using neuronal and astrocyte cell cultures from different brain regions of mice with perturbed DNA repair. Findings from these studies will be used to examine the *in vivo* neurotoxic effects of HN2 and MAM (Years 3 & 4) in mice with perturbed DNA repair.

BODY OF THE REPORT

STATEMENT OF WORK FOR YEAR 4 of FUNDING

The overall goal of studies proposed in Year 4 are to: (i) examine the DNA damaging properties of HN2 and MAM in neuronal cell cultures of DNA repair-mutant mice, (ii) use the results from the *in vitro* studies to begin dose-range finding studies of HN2 and MAM in wild type and DNA repair mutant mice, and (iii) examine the *in vivo* neurotoxic effects of HN2 and MAM in wild type and DNA repair mutant mice. Specific objectives proposed in Year 4 of the Statement of Work are as follows:

1. Complete DNA damage analysis of HN2 and MAM in $AAG^{-/-}$ neuronal and astrocyte cell cultures.
2. Complete DNA damage analysis of HN2 and MAM in $XPA^{-/-}$ neuronal and astrocyte cell cultures.
3. Complete DNA damage analysis of HN2 and MAM in $MGMT^{-/-}$ neuronal and astrocyte cell cultures.
4. Examine HN2- and MAM-treated $AAG^{-/-}$ mice for neuropathology.
5. Examine HN2- and MAM-treated $XPA^{-/-}$ mice for neuropathology.
6. Examine HN2- and MAM-treated $MGMT^{-/-}$ mice for neuropathology.

For the ongoing studies, we are currently breeding four strains of mice that either overexpress (1 strain) or are deficient (3-strains) in three key proteins of different DNA repair pathways. These are XPA, MGMT (1-overexpression, 1-knock-out), and AAG. The brain and peripheral organs (ears and kidneys) of wild type and DNA repair mutant mice were used to develop primary neuronal and astrocyte cell cultures or fibroblast and epithelial cell lines (*respectively*). The generation of $XPA^{-/-}$ mice appeared to be a particular problem with only 1 litter generated every 2 months during both Years 3 and 4. Therefore, *in vivo* neurotoxicity studies proposed in Year 4 were limited to the use of wild type, $MGMT^{-/-}$, $AAG^{-/-}$ and $MGMT^{+}$ mice. Skin fibroblast cell cultures and kidney epithelial cell cultures developed from each strain (i.e., $MGMT^{-/-}$, $AAG^{-/-}$, $XPA^{-/-}$) were used to clarify the acute (24h) and delayed (*cloning efficiency*) neurotoxic properties of HN2 and MAM. The balance of the animals (both wild type and DNA repair mutant) were used for dose-range finding studies and to examine for neuropathological changes. Despite significant problems encountered in the first three years of the grant, we were able to get back on 'track' and complete most of the objectives as proposed in Year 4 of the grant. However, we were still unable to generate a sufficient number of animals to complete the *in vivo* neurotoxicity studies (*especially with HN2*). Consequently, we applied for a no-cost extension and it was granted (9/02) to complete these *in vivo* studies. Therefore, wild type and the three DNA repair mutant mice were used primarily in Year 4 studies to: (i) complete the *in vitro* acute toxicity studies of HN2 and MAM, (ii) examine the *in vitro* delayed neurotoxic properties of HN2 and MAM and (iii)

examine the *in vivo* neurotoxic properties of HN2 and MAM. We were successful in examining all of the objectives of the Statement of Work (*except for # 2 & #5 that require XPA$^{-/-}$ mice*) in Year 4 by specifically focusing on: (i) the comparative delayed toxicity of HN2 and MAM in neuronal cultures from different DNA repair mutant and wild-type mice, (ii) the comparative toxicity of HN2 and MAM in non-nervous tissue (i.e., skin fibroblasts, kidney epithelial cells) of wild type and DNA repair mutant mice, (iii) the extent of DNA damage induced by HN2 and MAM in neuronal cultures from wild type and DNA repair mutant mice, and (iv) the extent of neuronal loss and degeneration induced by MAM in affected (cerebellum) and unaffected (midbrain) brain regions of wild type and DNA repair mutant mice. A detailed description of the research accomplishments for each objective of Year 4 follows.

1. Examine acute cytotoxicity of HN2 and MAM in neuronal cultures from DNA repair mutants.

Studies conducted in Year 3 of the grant demonstrated that murine cerebellar neurons were (i) more sensitive to HN2 and MAM than neurons from other brain regions (i.e., cortex, midbrain), (ii) more sensitive to HN2 and MAM than cerebellar astrocytes, and (iii) the sensitivity of HN2 and MAM treated wild type neurons correlated with an increase in markers of apoptosis and DNA damage (i.e. ,AP sites, apoptotic bodies). Since wild type astrocytes were relatively insensitive to both MAM and HN2, the remaining studies (Years 2-4) focused primarily on the neurotoxic properties of these DNA damaging agents. Since cerebellar neurons were the most sensitive CNS cell type to the acute toxic effects of MAM and HN2, cerebellar neuronal (granule cell) cultures were used throughout the remainder of the *in vitro* toxicity studies in Years 3 and 4. Granule cells are also a primary target of MAM when adminstered to neonatal rats or mice [4] and are, therefore, a viable neuronal cell type to explore the *in vitro* and *in vivo* neurotoxic effects of MAM and HN2.

Viability of DNA repair deficient neurons

The central hypothesis under study is that DNA damage is a primary mechanism of mustard-induced neuronal cell death. To test this hypothesis, neuronal cultures were prepared from mice that are proficient (wild type) or deficient (AAG$^{-/-}$, MGMT$^{-/-}$, XPA$^{-/-}$) in DNA repair, the cultures treated for 24h with HN2 and MAM and examined for cell survival using the well-established vital fluorochromes calcein-AM and propidium idodide (*see* **Figure 1**, Appendix). In general, the survival of mature post-mitotic neurons was significantly reduced with increasing concentration of HN2 or MAM. However, AAG$^{-/-}$ cerebellar neurons appeared relatively insensitive, especially at high concentrations of HN2 (>5 μM) or MAM (>500 μM). Although these results were unexpected, our findings are consistent with recent studies demonstrating that bone marrow cells of AAG$^{-/-}$ mice are resistant to the cytotoxic effects of alkylating agents [13]. The basis for this protection is reported to be an imbalance in enzymes of the base-excision DNA repair pathway [12]. In contrast, MGMT$^{-/-}$ and XPA$^{-/-}$ neurons were more sensitive to HN2 or MAM than similarly treated wild type or AAG$^{-/-}$ cells. The increased sensitivity of MGMT$^{-/-}$ neurons to MAM is consistent with the production of O^6-methylguanine DNA adducts, a minor DNA adduct (~1-6%) generated by this genotoxin.

MGMT^{+} Studies

The above studies indicate that MGMT$^{-/-}$ neurons were particularly sensitive to the acute toxic effects of both MAM and HN2. Although MAM is known to generate multiple DNA adducts (i.e., N^7-methylguanine, O^6-methylguanine, 8-methylguanine), the above studies suggest that O^6-methylguanine DNA adducts are the primary DNA lesion responsible for the acute neurotoxic effects of MAM. The increased sensitivity of MGMT$^{-/-}$ neurons to HN2 is, however, less clear since mustards are not known to produce DNA adducts that are repaired by MGMT. However, recent studies suggest there is

considerable cross-talk among DNA repair pathways [6,12] and this could account for the increased sensitivity of $MGMT^{-/-}$ neurons to HN2. Therefore, if $MGMT^{-/-}$ neurons are indeed sensitive to both HN2 and MAM, then we would expect that neurons that overexpress MGMT ($MGMT^{+}$) should be protected from the acute toxicity of both of these genotoxins. To test this hypothesis, $MGMT^{+}$ and $MGMT^{-/-}$ cerebellar neurons (*see* **Figure 2,** Appendix) were treated with similar concentrations of HN2 and MAM and examined for cell viability by measuring mitochondrial function (Alamar Blue™) and the live/dead assay (calcein AM/propidium iodide). In comparison to $MGMT^{-/-}$ or wild type neurons (*see* **Figure 1**, Appendix), $MGMT^{+}$ neurons were partially protected from the acute toxic effects of both MAM and HN2. However, significant protection was only observed at high concentrations of MAM (>500 μM) while survival was higher for $MGMT^{+}$ neurons at all concentrations of HN2 tested, though only the protection was only partial. These findings suggest that O^6-methylguanine DNA adducts play an important role in the acute neurotoxicity of MAM and MGMT provides protection, by an unknown mechanism, against HN2-induced neurotoxicity. This protection may be mediated by a different mechanism, possibly by its influence on other DNA repair pathways (e.g., base-excision or nucleotide excision DNA repair pathways).

2. DNA Damage in HN2 and MAM Treated DNA Repair Mutant Neuronal Cultures.

Findings from the above $MGMT^{-/-}$ and $MGMT^{+}$ studies suggest that O^6-methylguanine DNA adducts play a major role in the acute neurotoxic effects of MAM, but the exact mechanism of HN2 is unknown. The basis for this increased sensitivity is likely to be an increase in unrepaired damage to DNA induced by MAM or HN2. Inefficient removal of DNA damage induced by HN2 or MAM can lead to strand breaks and ultimately cell death. To clarify the relationship between DNA damage and the increased sensitivity of $MGMT^{-/-}$ neurons to these agents, we compared the extent of DNA damage (i.e., strand breaks) induced by HN2 and MAM in wild type, $AAG^{-/-}$, $MGMT^{-/-}$, and $MGMT^{+}$ neurons. To test this hypothesis, cerebellar neurons from wild type and DNA repair mutant ($AAG^{-/-}$, $MGMT^{-/-}$, and $MGMT^{+}$) mice were plated at the same density (140-150K cells/well), treated for 24h with the same concentrations of MAM (10 μM, 100 μM, 1000 μM) or HN2 (1.0 μM, 5.0 μM, 10 μM) and the paraformaldehyde fixed cells examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.), which detects DNA strand breaks by the terminal deoxynucleotidyl trnasferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (*see* **Figure 3,** Appendix). In general, DNA damage increased with the concentration of either HN2 or MAM among all four genotypes. Cell counts were only available for MAM and HN2 treated wild type, $MGMT^{-/-}$ and $MGMT^{+}$ cerebellar neurons and the results are consistent with the previous viability studies (*see* **Figures 1 & 2**). As expected, DNA damage was significantly higher in $MGMT^{-/-}$ neurons treated with MAM than comparably treated wild type ($p < 0.01$) or $MGMT^{+}$ ($p < 0.001$) neurons. The pronounced protective effect of $MGMT^{+}$ on MAM-induced DNA damage and neuronal viability (*see* **Figure 2**), and later on in MAM-induced cerebellar degeneration (*see* **Figure 13**), is strong evidence in favor of the formation of O^6-methylguanine DNA adducts (*vs.* N^7-methylguanine DNA adducts) in MAM-induced neurotoxicity. Unlike MAM, $MGMT^{+}$ did not protect neurons against HN2-induced DNA damage even though viability studies indicate that cell survival was significantly higher in $MGMT^{+}$ neurons when compared to similarly treated $MGMT^{-/-}$ or wild type neurons. However, close examination of the previous viability studies indicates that $MGMT^{+}$ only provided partial protection since substantial (~50%) cell loss was observed at high concentrations of HN2. In contrast, the viability of MAM treated $MGMT^{+}$ neurons was ~3x higher than comparably treated $MGMT^{-/-}$ neurons. These findings would suggest that HN2 (*vs.* MAM) may induce cell death by multiple pathways (e.g., alkylation-induced DNA damage, oxidative stress) and MGMT

only provided partial protection from the genotoxin. Additional studies are underway to clarify the mechanism of HN2-induced neurotoxicity by examining the role of other DNA repair pathways (e.g., recombination, oxidative) or antioxidant enzymes (e.g., GSH).

HN2-Induced DNA Damage

The above DNA damage studies with HN2 revealed the possible complex mechanism(s) by which this genotoxic agent may induce acute neurotoxicity. To further explore the role of DNA damage (i.e., cross-links) in HN2-induced neurotoxicity, we compared the sensitivity of wild type (C57BL/6) cerebellar neurons to mechlorethamine (HN2) and its monofunctional mustard analogue 2-chloroethylamine (CEA) (*see* **Figure 4**, Appendix). Unlike HN2, CEA does not form cross-links with DNA and, consequently, is reportedly significantly less genotoxic and mutagenic than HN2 [16]. Therefore, CEA would predominantly alkylate nitrogen atoms in DNA of neurons to produce monoadducts (e.g., N^7-alkylpurines) and not cross-links. Mouse cerebellar neurons were treated with similar concentrations (1.0 μM, 5.0 μM, 10 μM, 20 μM) of HN2 or CEA and cell survival determined 24h later by the live/dead assay and Alamar Blue™ (*for details see* **Figure 1**). Cell survival was significantly higher in wild type cerebellar neurons that were treated with CEA than after HN2 treatment. These studies suggest that monoadducts produced by CEA and HN2 are efficiently repaired by wild type neurons (*which are produced by both agents*), but that the ability of HN2 to form cross-links is likely responsible for the increased sensitivity of neurons to HN2. Comparable studies are currently planned for $MGMT^{-/-}$ and $MGMT^{+}$ cerebellar neurons to determine if a similar pattern of sensitivity occurs for CEA and HN2. Findings from these studies may help clarify the difference we observed between the viability and the extent of DNA damage after HN2 treatment of $MGMT^{+}$ neurons.

Oxidative Stress

The above viability studies with MAM and HN2 demonstrate that $MGMT^{-/-}$ and $XPA^{-/-}$ neurons are particularly sensitive to these genotoxic agents, possibly by the generation of O^6-methylguanine DNA adducts or cross-links, respectively. However, the influence of MAM and HN2 on mitochondrial function (i.e., Alamar Blue™) and their reported generation of reactive oxygen species [9] or influence on cellular antioxidant enzymes (e.g., GSH) [11] suggests that the increased sensitivity of these cells to HN2 and MAM may be occur through oxidative stress. To further explore the role of oxidative stress-induced neuronal cell death mediated by HN2 and MAM, we compared the sensitivity of cerebellar neurons from wild type, $MGMT^{-/-}$ and $XPA^{-/-}$ mice after exposure to the oxidant menadione, a cytotoxic agent that produces cell death through the generation of free radicals (e.g., superoxide) [1]. Mouse cerebellar neurons from wild type, $MGMT^{-/-}$ and $XPA^{-/-}$ mice were treated with menadione (1.0 μM, 10 μM, 50 μM) and cell survival determined 24h later by measuring fluorescence *via* the Alamar Blue™ assay (*see* **Figure 5**, Appendix). Alamar Blue™ is a non-toxic metabolic indicator that is widely used to measure mitochondrial function in different cell systems (including neurons) [14,15]. Redox activity was not significantly different between menadione treated neurons from wild type or DNA repair mutant mice except at the highest concentration of the oxidant for $XPA^{-/-}$ neurons. These studies suggest that the acute neurotoxic effects of MAM and HN2 are not primarily mediated through oxidative stress induced cell death. Additional studies are underway to confirm these findings by examining glutathione levels in HN2 and MAM treated cerebellar neurons from wild type and DNA repair mutants.

3. Delayed Cytotoxicity of HN2 and MAM in DNA Repair-Deficient Neural Cultures.

The notion that the persistence of DNA adducts may be associated with delayed neurotoxicity is supported by the increased sensitivity of neurons (*see* **Figure 1**, Appendix) or fibroblasts and epithelial cells (*Year 3 Studies*) from $MGMT^{-/-}$ and $XPA^{-/-}$ after a brief (24h) exposure to MAM or HN2. The insufficient number of $XPA^{-/-}$ mice for these experiments limited the delayed studies to wild type, $AAG^{-/-}$ and $MGMT^{-/-}$ mice. Therefore, additional studies were conducted with wild type, $AAG^{-/-}$, and $MGMT^{-/-}$ mice to determine if the increased sensitivity of $MGMT^{-/-}$ neurons to HN2 and MAM also occurs after prolonged exposure (*up to 7 days*) to the genotoxins (*see* **Figure 6**, Appendix). Compared to wild type and $AAG^{-/-}$ neurons, $MGMT^{-/-}$ neurons were more sensitive to low concentrations of HN2 (>1.0 μM) and MAM (>100 μM) and cell loss increased with time. These studies demonstrate that high concentrations of mustards and MAM are acutely toxic to neurons while low concentrations induce a delayed neurotoxicity. The unexpected sensitivity of $MGMT^{-/-}$ neurons to HN2 suggests that mustards either produce O^6-methylguanine DNA adducts or cellular pathways that repair HN2-induced cross-links (e.g., NER, recombination, mismatch) are also perturbed in $MGMT^{-/-}$ neurons. Additional studies are underway to determine if these repair pathways play an important role in HN2-induced neurotoxicity.

4. Cytotoxicity of HN2 and MAM in DNA Repair-Deficient Non-Neural cultures.

Viability Studies

The above studies demonstrate that the targeted reduction of DNA repair (i.e., $MGMT^{-/-}$, $XPA^{-/-}$) within neurons increases their sensitivity to HN2 and MAM, possibly *via* the production of specific DNA adducts (e.g., O^6-methylguanine, cross-links). However, non-neural tissues (e.g., skin) are one of the primary targets of mustards [5] and, therefore, the action of these agents on non-neural tissues may also occur by a similar mechanism. In Year 3 studies, we demonstrated that fibroblast cultures developed from DNA repair mutant mice exhibited a differential sensitivity to MAM and HN2. In preliminary short-term studies (24h), $MGMT^{-/-}$ and $XPA^{-/-}$ cells appeared particularly sensitive to MAM and HN2. Long-term studies with fibroblasts revealed a similar pattern of vulnerability to HN2 and MAM. Additional studies were conducted with kidney epithelial cells from wild type, $AAG^{-/-}$, $MGMT^{-/-}$, and $XPA^{-/-}$ mice to confirm the previous fibroblast findings after exposure to HN2 and MAM and to determine if a similar pattern of vulnerability occurs among different tissues. To test this hypothesis, fibroblast (*see* **Figure 7,** Appendix) and epithelial (*see* **Figure 8**, Appendix) cell lines were prepared from the ears and kidneys of all three DNA repair-deficient mice (i.e., $AAG^{-/-}$, $MGMT^{-/-}$, $XPA^{-/-}$) and examined for their sensitivity to HN2 or MAM. A similar pattern of sensitivity emerged for skin fibroblasts and kidney epithelial cells treated with HN2 or MAM for 24h and the cells examined for survival 2-3 weeks later (*cloning efficiency*). Like neurons, skin fibroblasts and kidney epithelial cell cultures from $AAG^{-/-}$ mice were relatively insensitive to HN2 and MAM, while similarly treated cells from $MGMT^{-/-}$ and $XPA^{-/-}$ mice were sensitive to both genotoxins. However, $XPA^{-/-}$ skin fibroblasts appeared more sensitive to HN2 and $AAG^{-/-}$ kidney epithelial cells appeared more sensitive to MAM than comparably treated kidney or fibroblast cell cultures, respectively. A possible explanation for this differential response is that individual cell types may display complex phenotypic differences with respect to the cellular repair of DNA damage induced by alkylating agents [13].

MAM Induced DNA Damage

The above viability studies in $AAG^{-/-}$, $MGMT^{-/-}$, $XPA^{-/-}$ fibroblast and kidney epithelial cell lines and neuronal cultures indicate O^6-methylguanine DNA adducts play an important role in MAM-induced cell death. Cells or tissues that lack mismatch DNA repair are noted to be tolerant to alkylating agents that produce O^6-methylguanine DNA adducts. Cells that are proficient in mismatch DNA repair convert the base opposite to the O^6-methylguanine DNA lesion to a thymidine and this results in the repetative insertion and production of nicks (*futile cycle of repair & damage*)[8]. To further explore the role of O^6-methylguanine DNA adducts in MAM-induced cytotoxicity, kidney epithelial cells from wild type and mistmatch DNA repair-deficient (i.e., $PMS2^{-/-}$) mice were treated with MAM for 24h and examined for redox function (*see* **Figure 9**, Appendix). As expected, mismatch DNA repair deficient epithelial cells were significantly more resistant to the cytotoxic effects of MAM than similarly treated wild type cells. These studies provide additional strong evidence that MAM-induced cyototoxicity occurs through the generation of O^6-methylguanine DNA adducts.

5. Dose-Range Finding Studies of HN2 and MAM in DNA Repair-Mutant Mice.

Evidence from the acute and delayed toxicity studies of neuronal cell cultures with $MGMT^{-/-}$, $AAG^{-/-}$, and $MGMT^{+}$ mice conducted in Years 1-3 suggest we should begin dose-range findings studies with these three DNA repair mutant mice. Initial dose-range findings studies (Year 3) were first conducted in wild type mice by treating 3 day day old pups (*from two litters*) with three different doses of MAM: a high (43 mg/kg), moderate (21.5 mg/kg) or low dose (4.3 mg/kg). Results from these studies indicate that MAM reduces body weight and size (length from crown to rump) as previously reported with only a loss of 2 animals in the high dose group. Additional studies were conducted in Year 4 to continue examining wild type and DNA repair mutant ($MGMT^{-/-}$, $AAG^{-/-}$, and $MGMT^{+}$) mice for viability and neuropathology (*see* **Figures 10-13**) after a single subcutaneous dose of MAM (high, moderate, low doses used in Year 3) or HN2 (high: 40 mg/kg, 20 mg/kg, 10 mg/kg; moderate: 5 mg/kg and 2.6 mg/kg; low: 1.3 mg/kg) in saline or a similar volume of saline (control). The dose and treatment age for both genotoxins were chosen based upon several important factors: (i) the cerebellum (notably the granule cell layer) is severely compromised in mouse pups treated at 1-5 days with MAM [2,7], (ii) neuronal cultures from the cerebellum of wild type and $MGMT^{-/-}$ were especially sensitive to HN2 (*see* **Figures 1,2,4**, Appendix) and (iii) $MGMT^{+}$ partially protected cerebellar granule cell cultures from MAM- and HN2-induced neurotoxicity (*see* **Figure 2,** Appendix). The animals were examined daily for changes in body weight and size and periodically observed (*weekly*) for signs of motor dysfunction (hindlimb splay, ataxia, lethargy), features that typically are reduced in MAM treated animals. As previously reported, the body weight of MAM (i.e., 43 mg/kg and 21.5 mg/kg) treated animals were typically 20-25% lower than saline treated littermates for both wild type and DNA repair deficient mice (i.e., $MGMT^{-/-}$, $AAG^{-/-}$) and remained lower up until termination at day 22. Similar results were obtained for wild type and DNA repair deficient mice (i.e., $AAG^{-/-}$) treated with HN2 (10 mg/kg, 5 mg/kg, 2.6 mg/kg). Body weights for the low MAM or HN2 dosed animals were similar to saline treated animals. HN2 was particularly toxic to both wild type and $AAG^{-/-}$ mice at doses > 10 mg/kg with 100% of the animals dying within 3 days of dosing and 50% of the animals dying (LD_{50}) at 5 mg/kg. However, 100% of wild type and $AAG^{-/-}$ mice lived at lower concentrations of HN2 (2.6 mg/kg, 1.3 mg/kg) or MAM (4.3 mg/kg). $MGMT^{-/-}$ and $MGMT^{+}$ mice were only dosed with MAM and LD50 determined to be 21.5 mg/kg for $MGMT^{-/-}$ mice while none of the MAM treated $MGMT^{+}$ mice died before termination (22 days). The LD50 for MAM in wild type mice was determined to be 43 mg/kg while 100% of the $MGMT^{-/-}$ mice died at this dose of the genotoxin. These studies indicate that wild type, $AAG^{-/-}$ and $MGMT^{-/-}$ mice are very sensitive to high concentrations of HN2 and MAM with a majority (>50%) of

the animals living either at moderate or low concentrations of the genotoxins. Results from these dose-range findings studies are consistent with our previously reported sensitivity of $MGMT^{-/-}$ neurons to MAM or, conversely, the increased protection of $MGMT^{+}$ neurons from MAM-induced toxicity. The increased sensitivity of $AAG^{-/-}$ mice to high concentrations of MAM may be explained by the increased sensitivity of β-islets to alkylating agents [3]. Results from the HN2 studies are inconclusive at this time until additional studies are conducted (*no-cost extension*) with wild type and DNA repair mutant mice.

6. **Neuropathology of Brain Tissue from MAM Treated DNA Repair-Mutant Mice.**

Persistent DNA damage, either from excessive alkylation and/or reduced DNA repair, is a possible mechanism by which HN2 or MAM could have persistent effects on CNS function. Previous studies demonstrate that MAM disturbs neuronal development within the cerebellum of 1-5 day old neonatal mice [2,7]. Since the above *in vitro* studies demonstrate that MGMT protects cerebellar neurons from MAM-induced cell death, additional studies were conducted to determine if this DNA repair protein also protects *in vivo* developing cerebellar neurons from MAM. Wild type (C57BL/6), $MGMT^{-/-}$ and $MGMT^{+}$ 3-day old neonatal (PND3) mice were injected with saline or MAM and, 24h later, the brain sectioned, stained and examined by light microscopy for neuropathology.

Animals were perfused with 4% buffered paraformaldehyde and the brain and spinal cord cryoprotected in sucrose. Sagittal brain tissue sections were made through the whole cerebellum, the serial sections stored at –90°C in cryoprotectant, and every tenth section examined for cerebellar morphology (cresyl violet **[A]** or anti-calbindin-D **[B]** staining), neurodegeneration (silver staining, **[C]**) or dopaminergic neurons (i.e., anti-tyrosine hydroxylase, **[D]**). Calbindin-D is an intracellular calcium-binding protein that is especially abundant in Purkinje cells of the cerebellum and is, thus, a very useful marker for Purkinje cell degeneration. Components of neurons undergoing degeneration (e.g., lysosomes, axons, terminals) become agryrophilic (affinity for silver ions) and upon reduction form dark grains that are visible by light microscopy. Gross observation of the cerebellum from MAM treated wild type mice revealed extensive atrophy of the cerebellar lobes (*stars*) when compared with the cerebellum of saline treated mice (*see* **Figure 10A & B**, Appendix). This was more evident in cresyl violet stained (*see* **Figure 12A**, Appendix) and anti-calbindin immunoprobed (*see* **Figure 12B**, Appendix) sagittal sections of the cerebellum from MAM treated $MGMT^{-/-}$ mice. These stains revealed extensive hypogranulation of the cerebellum and the disorganization and displacement of neurons within both the granule (GL) and Purkinje (PL) cell layers. Particularly noticeable was the heavy deposition of silver stain (*green arrows*) over neurons within the molecular layer of the cerebellum (*see* **Figure 12C**, *Inset*) an indication that these cells are injured or damaged. MAM was also observed to have additional effects on the midbrain (i.e., substantia nigra, SN) of treated mice, a brain region not known to be affected by MAM. Tyrosine hydroxylase immunoreactivity was noted to be reduced in SN neurons of wild type mice treated with MAM (*see* **Figure 10D**, Appendix), but this effect was more evident in $MGMT^{-/-}$ mice treated with a 2-fold lower dose of MAM (*see* **Figure 12D**, Appendix). Comparable studies with either $AAG^{-/-}$ mice (*see* **Figure 11**, Appendix) or $MGMT^{+}$ mice (*see* **Figure 13**, Appendix) demonstrated that the cerebellum and midbrain from these animals were less perturbed by MAM. These latter findings with $AAG^{-/-}$ and $MGMT^{+}$ mice are consistent with the previously reported protection of neuronal cultures derived from these mice treated with MAM.

These *in vivo* findings with MAM in $MGMT^{-/-}$, $AAG^{-/-}$, and $MGMT^{+}$ mice are consistent with the findings from our previous *in vitro* studies with the same mutant mice. Therefore, we provide strong *in vitro* and *in vivo* evidence that MAM induces its neurotoxic effects *via* the generation of O^6-

methylguanine DNA adducts, data which supports our original hypothesis. Comparable studies are currently underway with $MGMT^{-/-}$, $AAG^{-/-}$, and $MGMT^{+}$ mice administered HN2 to determine if the extent of cerebellar and midbrain neuronal loss, neurodegeneration and DNA damage differs (or is similar) to that of MAM treated DNA repair mutant mice. These studies will be our primary focus in the no-cost extension period.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that neurons deficient in AAG are equally sensitive to the acute toxic effects of MAM or HN2 as wild type cells.
- Demonstrated that neurons deficient in MGMT and XPA are more sensitive than wild type cells to the acute toxic effects of MAM or HN2.
- Demonstrated that $MGMT^{+}$ neurons are relatively insensitive to MAM and HN2-induced toxicity.
- Demonstrated that the extent of DNA damage differs for HN2 and MAM among DNA repair deficient neurons (i.e., $MGMT^{-/-}$ *vs.* $MGMT^{+}$)
- Demonstrated that HN2 targets neurons *via* a DNA damage (i.e., cross-links) mechanism.
- Demonstrated that the long-term survival (i.e., cloning efficiency) of MAM and HN2 treated non-neural cells is dependent upon the DNA repair capacity of cells, evidence that supports a common mechanism of MAM- and HN2-induced DNA damage among tissues.
- Demonstrated that *in vivo* MAM induces severe loss and neurodegeneration of DNA repair-deficient cerebellar neurons and extensive disorganization of the cerebellar cytoarchitechture.
- Demonstrated that MAM induces severe reduction of tyrosine hydroxylase within dopaminergic neurons of the substantia nigra (SN) and disturbs the organization of SN neurons .

REPORTABLE OUTCOMES

1. Kisby, G.E., Wong, V., Olivas, A., Qin, X., Gerson, S.L., Samson, L., Turker, M.S. Neurons of DNA repair mutant mice are selctively vulnerable to DNA damage. Soc Neurosci Abstr 27:#967.4.

2. Y.W., Kow, Imhoff, B., Ingram, D.K., Kisby, G., Kohama, S.G. The influence of caloric restriction (CR) and age on base excision DNA repair (BER) in the rat brain. Soc Neurosci Abstr (*In press*). Findings from these studies examines the effect of age on brain tissue DNA repair and its influence by diet and will be presented at the SON Meeting on Nov 2-7, 2002 in Orlando, FL. (*partial support*)

3. Kisby, GE, Sproles, D., Pattee, P., Nagalla, SR. Gene expression profiling of the developing brain following treatment with methylazoxymethanol (MAM). Soc Neurosci Abstr (*In press*). Findings from these studies determine the impact of MAM on gene expression within affected and unaffected brain regions and will be presented at the SON Meeting on Nov 2-7, 2002 in Orlando, FL. (*partial support*)

4. Kisby, G.E., Lesselroth, H., Olivas, A., Wong, V., Samson, L., Turker, M.S. DNA repair protects neurons from genotoxin-induced cell death. J Neurochem (*In preparation*, see Appendix).

5. Kisby, G.E., Lesselroth, H., Olivas, A., Wong, V., Samson, L., Turker, M.S. The DNA repair protein O^6-methylguanine methyltransferase (MGMT) protects neurons from methylazoxymethanol (MAM)-induced cell death. (*In preparation*).

CONCLUSIONS

A central hypothesis under study in this grant is that the mechanism of neuronal cell death induced by mustards is initiated by DNA damage. Consequently, our primary objective was to use mouse models with perturbations in DNA repair (deficient and overexpressing) to clarify the molecular mechanisms by which mustards induce cell death or neural injury. For comparison, DNA repair mutant mice were also to be treated with methylazoxymethanol (MAM), an environmental agent that is strongly linked with a neurological disorder with features of Parkinson's disease and dementia. The selective vulnerability of neurons within the CNS is one of the key features of Parkinson's disease and related neurodegenerative disorders. Consistent with this notion, we demonstrate that DNA repair-deficient neurons (i.e., MGMT-/-, $XPA^{-/-}$) are selectively vulnerable to MAM and HN2 and that this vulnerablity differs, at least *in vitro*, for neurons. The mechanism underlying this regional and cell specific vulnerability was examined by comparing the acute and delayed neurotoxicity of HN2 and MAM in cerebellar neurons of mice with deficits in different cellular DNA repair pathways [i.e., direct reversal (MGMT), base-excision (AAG) and nucleotide excision (XPA)]. Findings from these studies indicate that DNA repair capacity/DNA damage is an important determinant of the vulnerability of neurons to the acute and delayed toxic effects of both HN2 and MAM. For example, $AAG^{-/-}$ cerebellar neurons were equally vulnerable as wild type cells to both MAM and HN2-induced neurotoxicity while other DNA repair deficient neurons (i.e., $MGMT^{-/-}$, $XPA^{-/-}$) exhibited an increased sensitivity to both genotoxins. Consistent with these findings, the long-term survival of $AAG^{-/-}$ fibroblasts and epithelial cells treated with HN2 or MAM was equal to or better than comparably treated wild type or DNA repair deficient fibroblasts (i.e., $MGMT^{-/-}$, $XPA^{-/-}$), respectively. DNA damage was examined in HN2 and MAM treated cerebellar neurons from wild type and DNA repair mutant mice to determine if there was a relationship between the extent and type of damage and acute neurotoxicity. Cerebellar neurons from wild type and DNA repair mutant mice were treated with HN2 and MAM and examined for strand breaks (TUNEL). These studies demonstrate that DNA damage preferentially accumulates in HN2 and MAM treated neurons and that the extent of DNA damage is dependent upon the efficiency of neuronal DNA repair (i.e., $MGMT^{-/-}$). However, the extent of DNA damage did not correlate with <u>acute</u> neurotoxic effects of HN2 for $MGMT^{+}$ neurons. One possibility is that the persistence of DNA adducts in HN2 treated $MGMT^{+}$ neurons may result from the perturbation of other cellular DNA repair pathways by overexpressing MGMT. Despite these findings, the relative insensitivity of both wild type and $MGMT^{-/-}$ cerebellar neurons to equimolar concentrations of the monofunctional nitrogen mustard 2-chloroethylamine suggests that cross-links are the primary DNA lesion responsible for HN2-induced neurotoxicity. Unexpectedly, neural and non-neural cells of $MGMT^{-/-}$ mice were especially sensitive to HN2, a protein whose primary function is to remove O^6-alkyl DNA adducts [10]. Since cells deficient in recombination repair or mismatch repair are particularly sensitive to HN2 [10], these cellular pathways may be distrupted in $MGMT^{-/-}$ neural or non-neural cells. Studies are currently underway with cells deficient in these DNA repair pathways to assess their contribution to HN2-induced neurotoxicity.

Comparative studies with MAM demonstrated that the generation of O^6-methylguanine DNA adducts is the primary event underlying MAM-induced *in vitro* and *in vivo* neurotoxicity. Particularly noteworthy, was the influence of MAM on the metabolism (i.e., tyrosine hydroxylase) and organization of neurons within the SN neurons and the degeneration of cerebellar neurons 3-4 weeks after toxin administration. These findings suggest that MAM can influence the integrity of dopaminergic neurons an effect that may be related to its role in the human neurological disorder western Pacific ALS/PDC. Additional studies are underway to confirm these findings and to determine if the effect on midbrain neurons from DNA repair mutant mice (i.e., $MGMT^{-/-}$) is related to an increase in DNA damage and progresses with age.

A comparison of the short-term (i.e., acute) and long-term (i.e., delayed) survival of HN2 and MAM-treated neuronal or non-neuronal tissue (i.e., fibroblasts, epithelial cells) in Year 4 studies has provided evidence to support our hypothesis that DNA damage is an important mechanism underlying the delayed neurotoxicity of these alkylating agents. Studies are now underway (*no-cost extension*) to confirm this hypothesis by examining the extent of DNA damage in long lived neuronal cultures from DNA repair mutant mice after brief (24h) or continuous exposure to HN2 or MAM (up to 2-3 weeks). Findings from these studies will complement the long-term survival studies conducted with fibroblasts and epithelial cells treated with HN2 and MAM in Year 4. DNA damage will be assessed on similarly treated neurons to determine if there is a correlation between the extent and type of DNA damage and the delayed neurotoxicity of HN2 and MAM. In parallel with these studies, DNA repair deficient mice will be administered HN2 and examined for neuronal loss, degeneration and DNA damage as previously conducted with wild type mice in Year 3 studies. Findings from these *in vitro* and *in vivo* studies should provide sufficient evidence to demonstrate that DNA damage is a primary mechanism of MAM- and HN2-induced neuronal cell death.

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APPENDIX

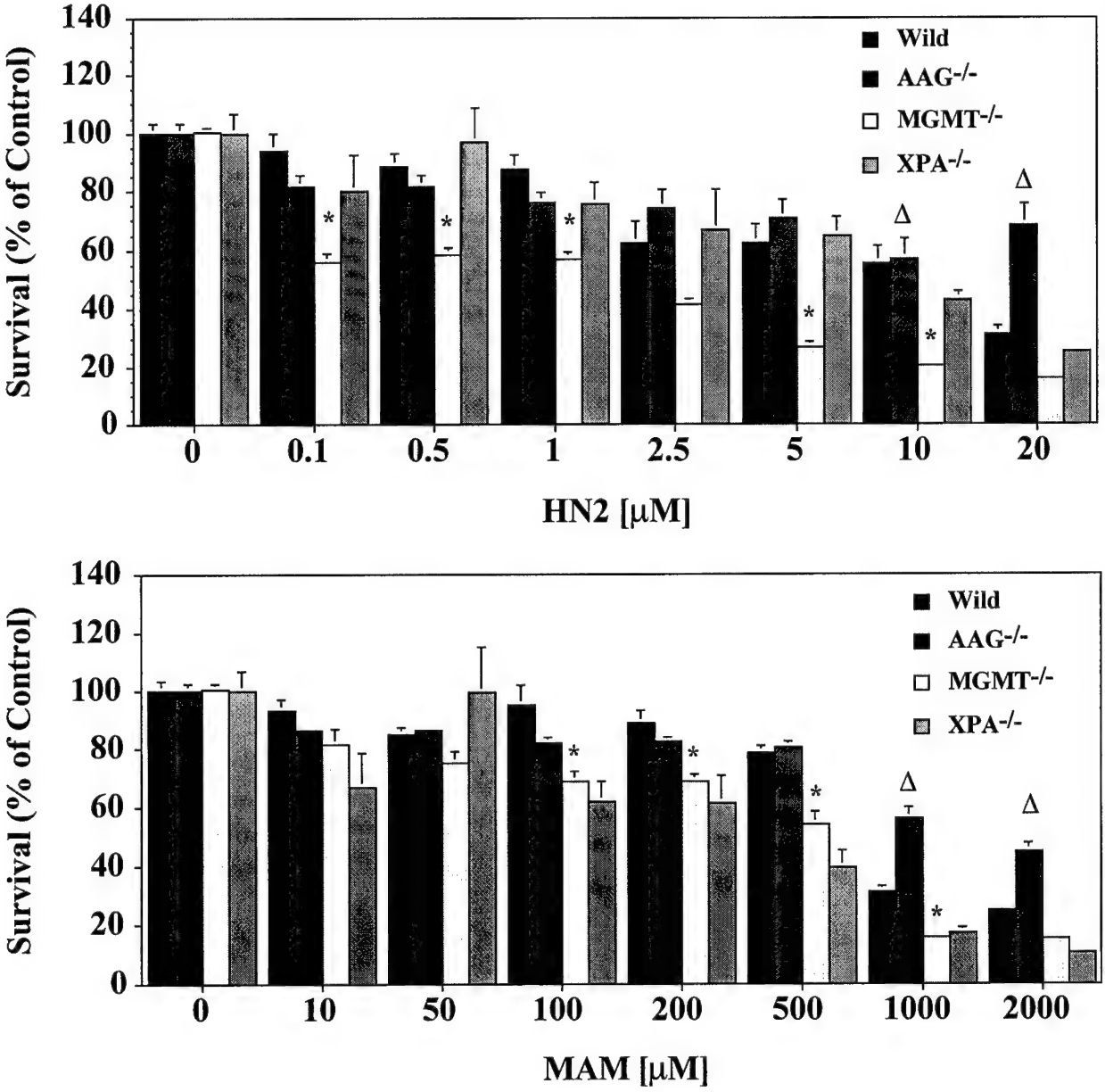

Figure 1. Viability of DNA Repair Deficient Cerebellar Neurons Treated with HN2 or MAM. Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) for 24h, incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM. Significantly different from toxin treated wild-type cells *(* $p < 0.01$, Δ $p < 0.001$,* ANOVA).

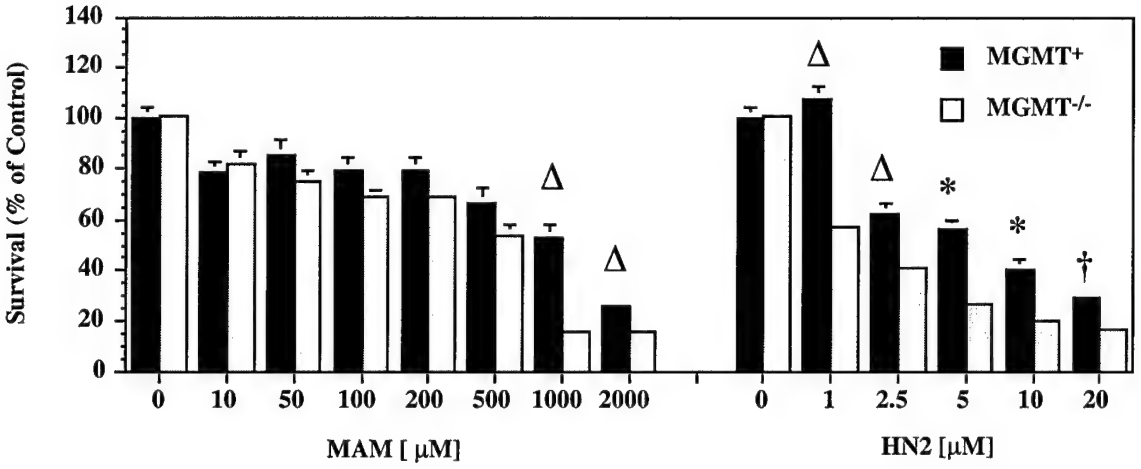

Figure 2. Viability of MGMT^{+} Neurons Treated with MAM or HN2. Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (1.0 μM-20 μM) for 24h, incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM. Significantly different from toxin treated MGMT$^{-/-}$ cells *(* $p < 0.05$, † $p < 0.01$, Δ $p < 0.001$*, ANOVA).

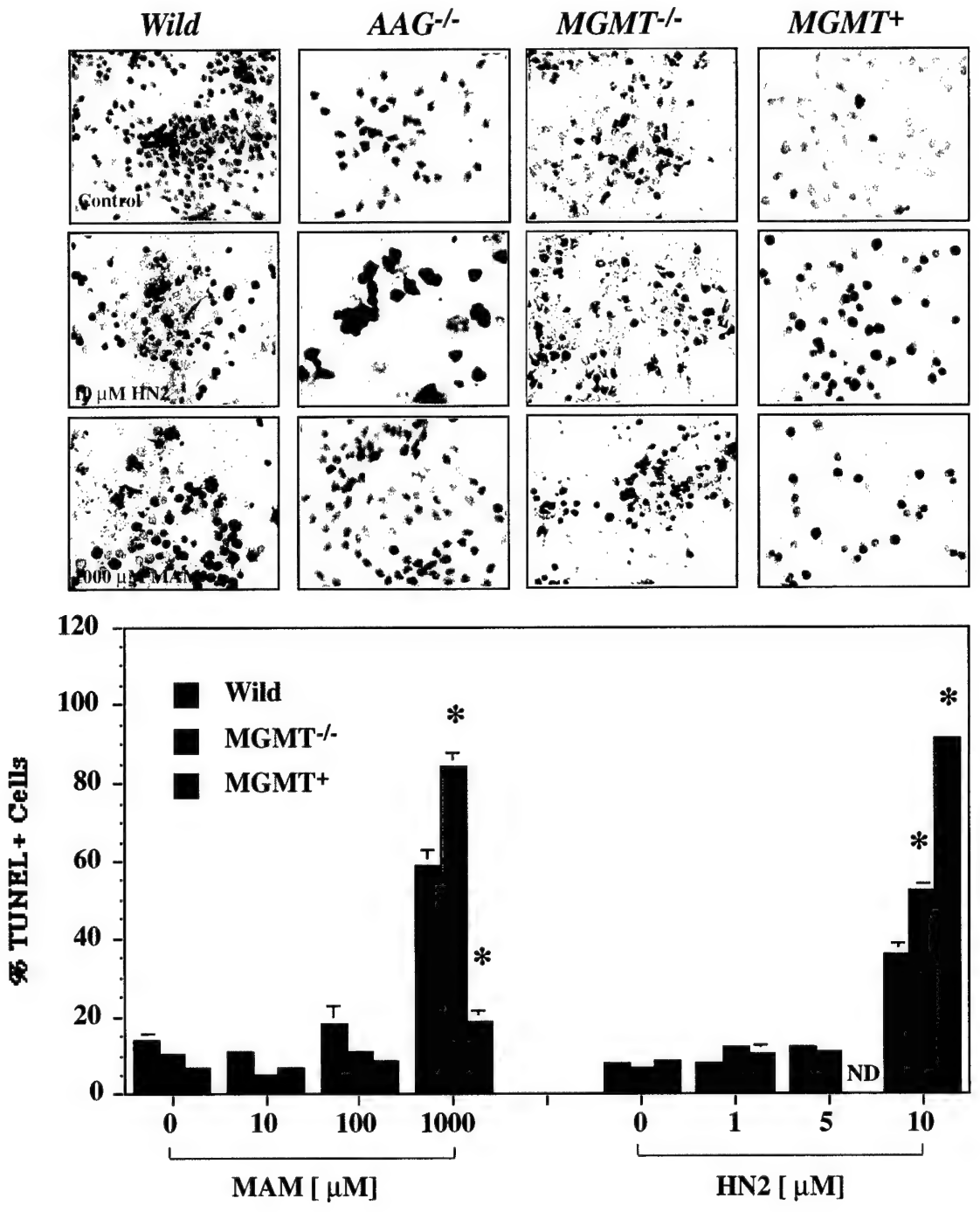

Figure 3. *In situ* DNA Damage of Wild Type (C57BL/6), $AAG^{-/-}$, $MGMT^{-/-}$ and $MGMT^{+}$ neurons Treated with MAM or HN2. Representative light micrographs of primary wild type DNA repair mutant cerebellar neurons that were treated for 24h with various concentrations of HN2 or MAM and examined for the extent of DNA strand breaks by TUNEL labelling (NeuroTacs" kit, Trevigen, Inc.). Note the extensive labeling of $MGMT^{-/-}$ neurons treated with 10 M HN2 and 1000 M MAM and the reduced labeling of MAM treated $MGMT^{+}$ neurons. Significantly different from wild type (* $p<0.01$, ANOVA) neurons.

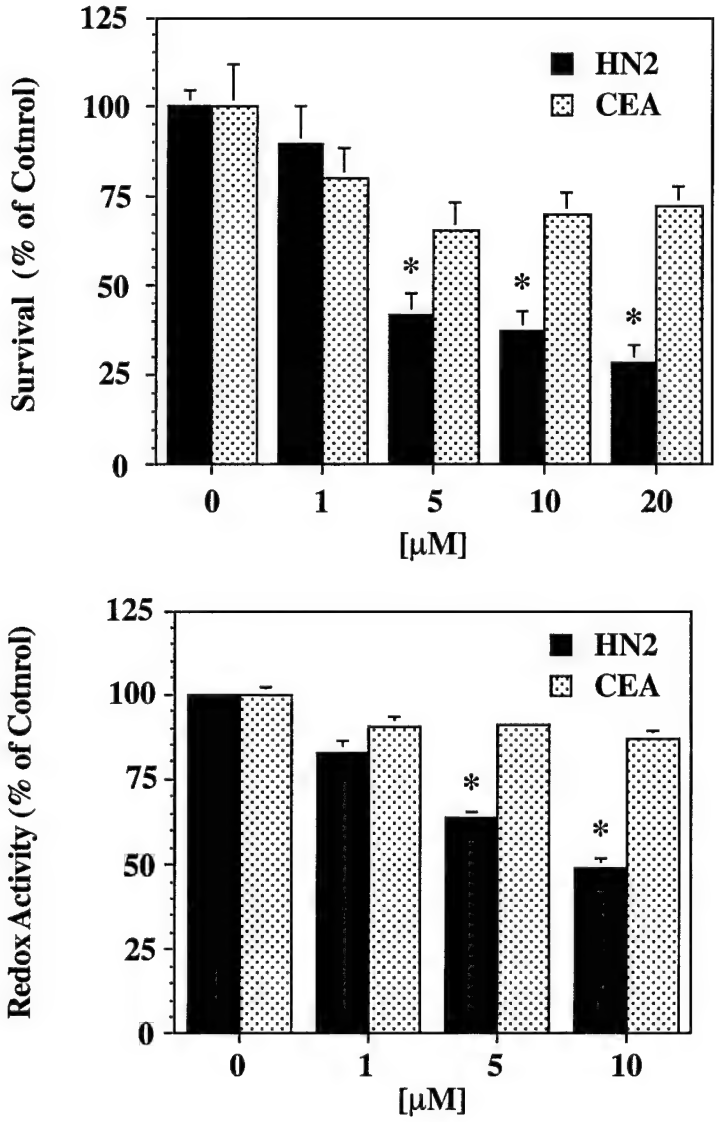

Figure 4. Viability of Wild Type Cerebellar Neurons Treated with mechlorethamine (HN2) or the monofunctional nitrogen mustard 2-chloroethylamine (CEA). C57BL/6 mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM-20 μM) or CEA (0.1 μM-20 μM) for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence (*bottom*). After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and the fluorescence measured by a microplate reader (*top*). Values represent the mean ± SEM (*n=6*, 2-3 separate experiments). Significantly different from CEA treated cells*(*p<0.001*,ANOVA).

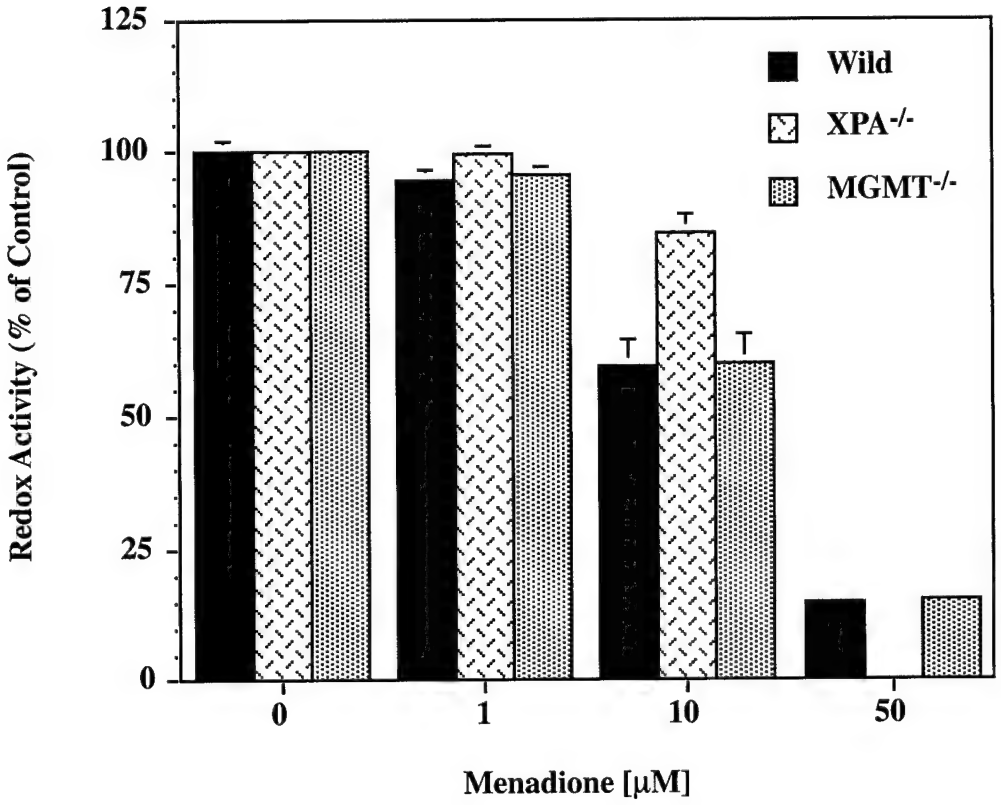

Figure 5. Viability of Cerebellar Neurons from DNA Repair-Deficient Mice Treated with the Oxidant Menadione. Mouse cerebellar granule cell cultures from wild type (C57BL/6), $MGMT^{-/-}$ and $XPA^{-/-}$ mice were treated for 24h with various concentrations of menadione (1.0 μM to 50 μM), the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. Values represent the mean ± SEM (*n=6*, 2-3 separate experiments).

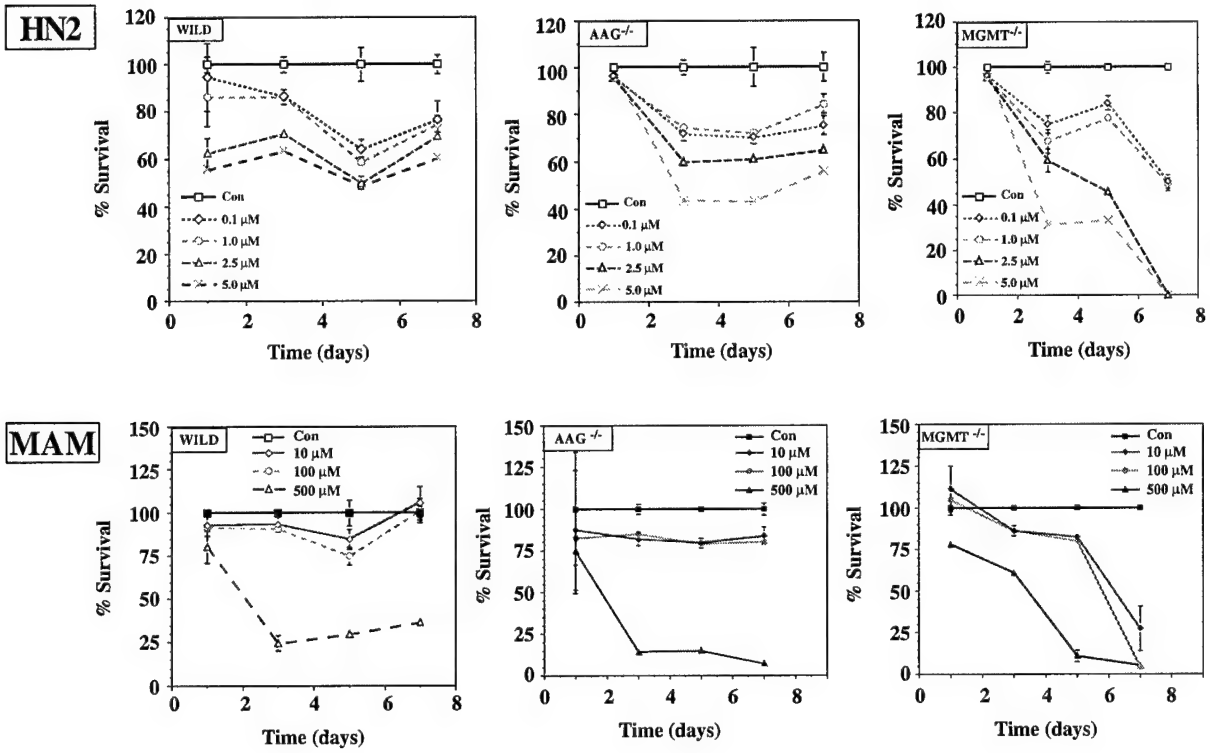

Figure 6. Long Term Viability of Neurons Treated with MAM or HN2. Mouse cerebellar granule cell cultures were treated continuously with HN2 (0.1 µM-5.0 µM) or MAM (10 µM-500 µM) and, at various time periods (1, 3, 5, 7 days), the cell cultures incubated with calcein-AM and examined for fluorescence. Values represent the mean % survival of controls ± SEM (*n= 6/group,* 2-3 separate experiments).

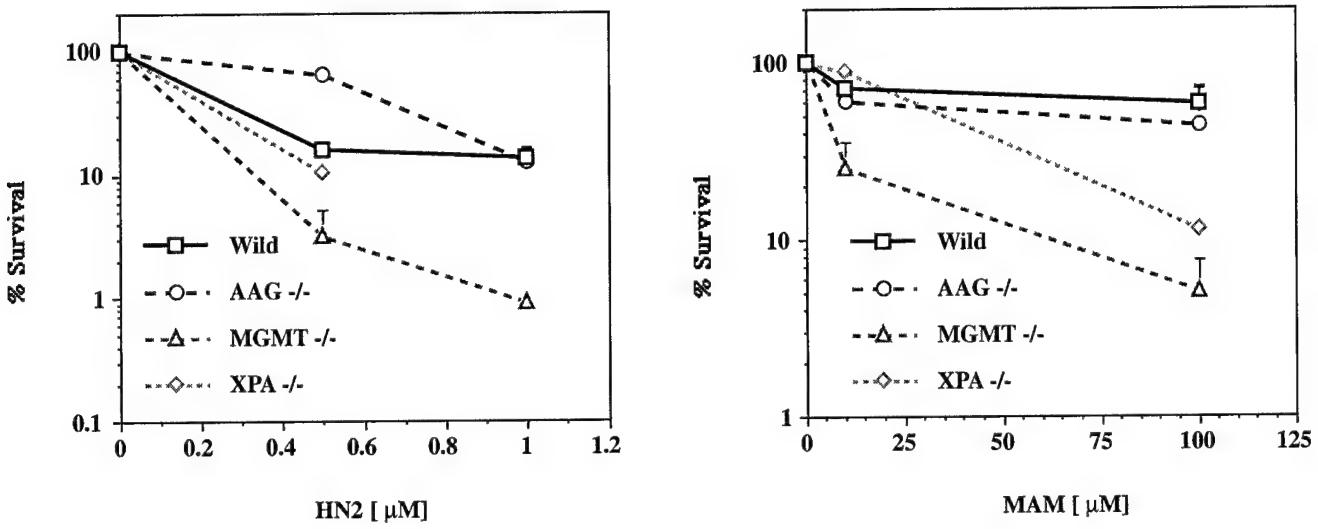

Figure 7. Cloning Efficiency of DNA Repair-Deficient Skin Fibroblasts Treated with HN2 or MAM. Cultures of mouse skin fibroblasts were treated for 24h with HN2 (0.1 μM-1.0 μM) or MAM (10 μM-1000 μM) and after 2-3 weeks *in vitro* (75-80% confluency), the cell cultures stained with cresyl violet and the number of colonies counted. Values represent the mean ± SEM from three separate experiments.

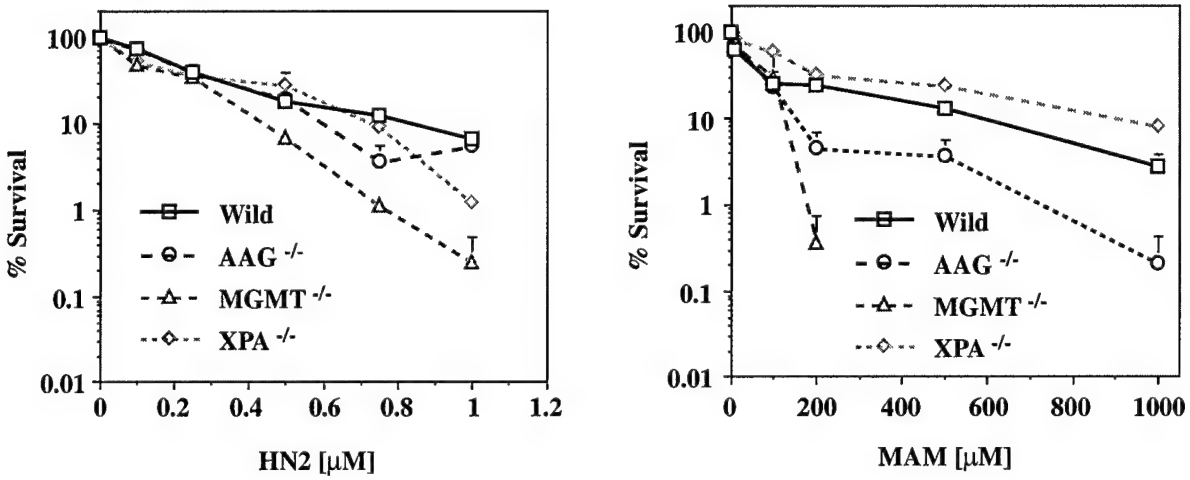

Figure 8. Cloning Efficiency of DNA Repair-Deficient Kidney Cells Treated with HN2 or MAM. Mouse kidney epithelial cell cultures were treated for 24h with HN2 (0.1 μM-1.0 μM) or MAM (10 μM and 1000 μM), the cell culture media replaced with toxin-free media and after 2-3 weeks *in vitro* (75-80% confluency), the cell cultures stained with cresyl violet and the number of colonies counted. Values represent the mean ± SEM from three separate experiments. Significantly different from HN2 or MAM treated wild-type cells.

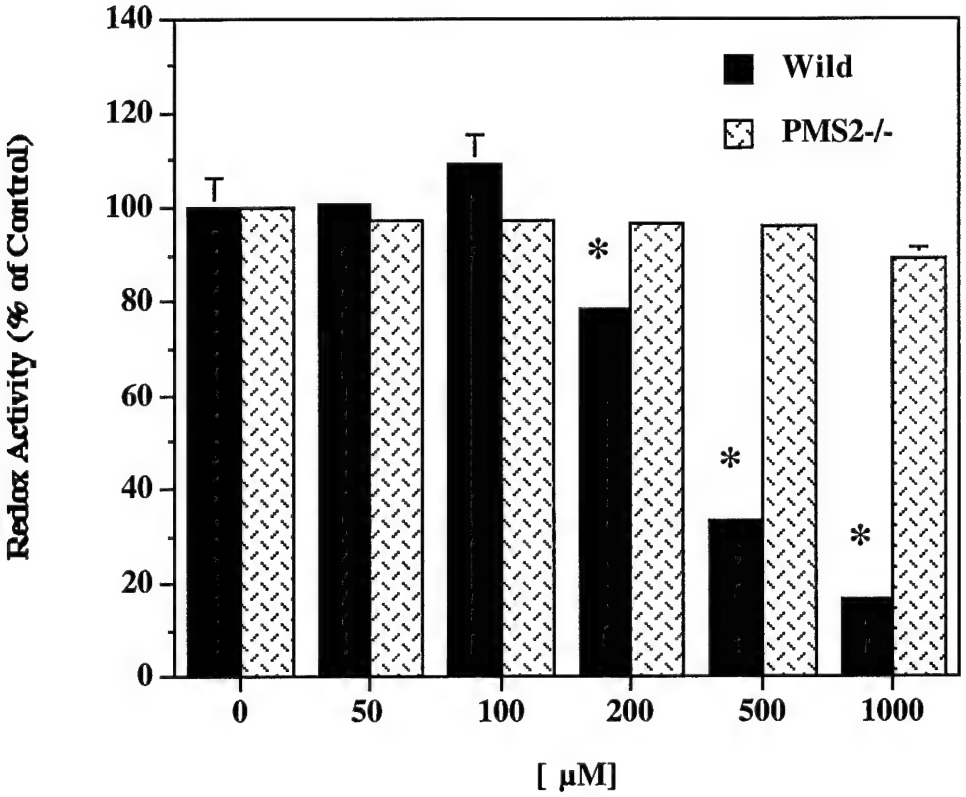

Figure 9. Viability of Mismatch DNA Repair-Deficient Kidney Epithelial Cells Treated with MAM. Mouse kidney epithelial cell cultures were treated for 24h with MAM (50 µM to 1000 µM), the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. Values represent the mean ± SEM (*n=6*, 2-3 separate experiments). Significantly different from wild type treated cells *(*p<0.0001*,ANOVA).

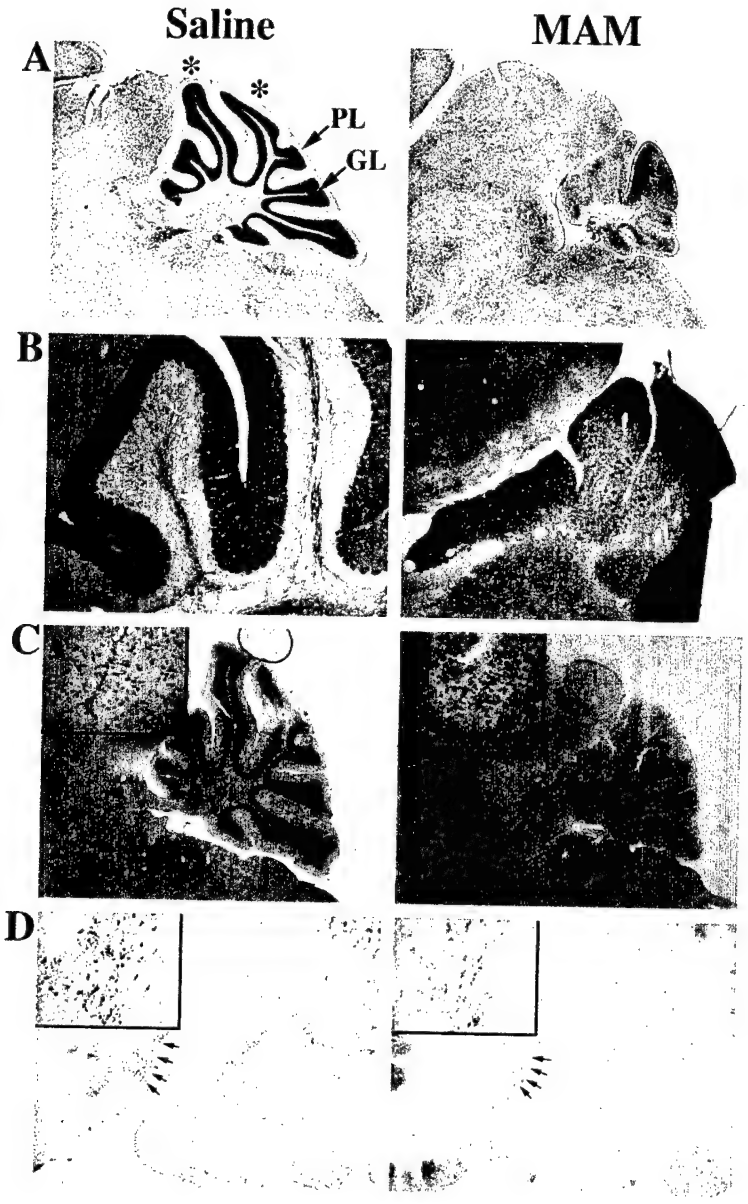

Figure 10. Neuropathology of the Brain from Postnatal C57BL/6 Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B,C**] or midbrain [**D**] of 22 day-old pups treated at postnatal day 3 with saline or MAM (43 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the smaller cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections. At the light microscopic level (**B**), smaller cerebellar folia (F), thinner cerebellar cortex and disorganization of neurons in the granule (GL), Purkinje (PL) and molecular (ML) layers were observed. CaBP was particularly useful for visualizing the disorganization and abnormal appearance of Purkinje cells within the cerebellum of MAM treated animals (**B**). Note also the reduced staining for tyrosine hydroxylase in nigral neurons (**D**) and the preservation of the neuronal organization (*vs.* $MGMT^{-/-}$ neurons, *compare with* **Figure 12D**) within the midbrain of MAM treated mice.

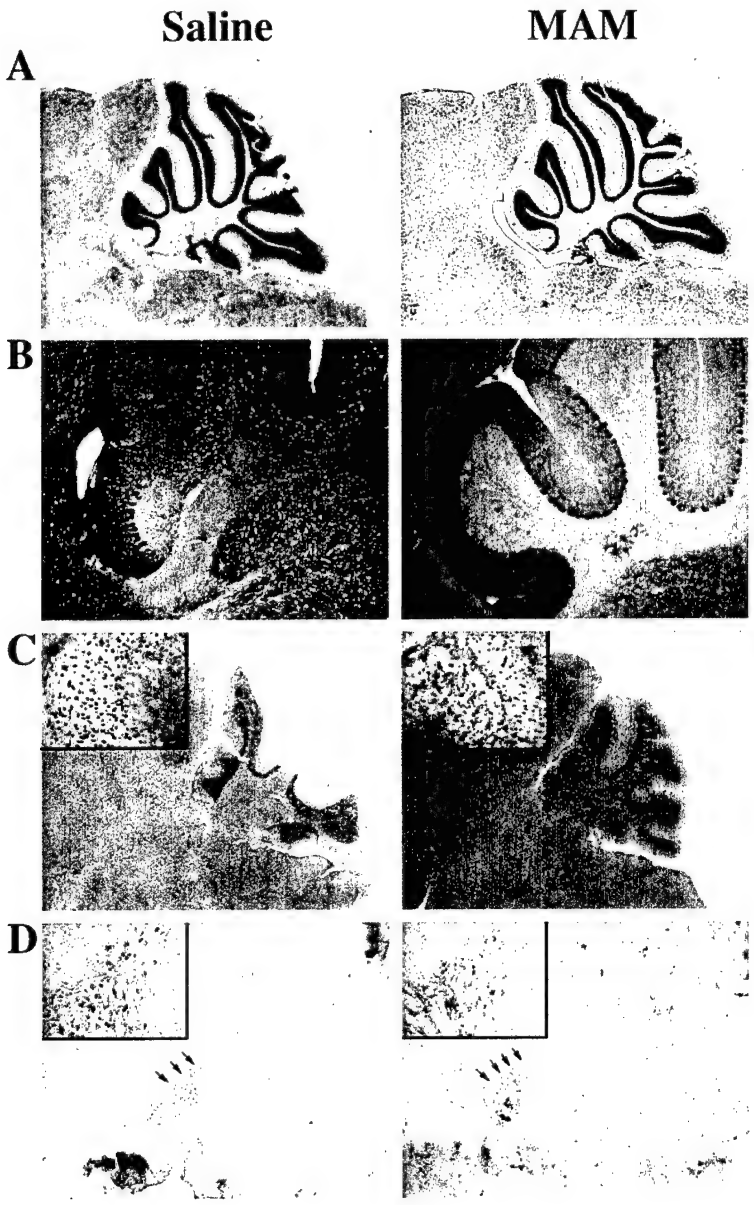

Figure 11. Neuropathology of the Brain from Postnatal AAG$^{-/-}$ Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B,C**] or midbrain [**D**] of 22 day-old pups treated at postnatal day 3 with saline or MAM (21.5 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the preservation of the cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections when compared to moderately dosed MGMT$^{-/-}$ mice (*see* **Figure 12A**). At the light microscopic level (**B**), the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in CaBP (**B**) and silver stained (**C**) tissue sections of MAM treated mice. Note also the organization and staining intensity of tyrosine hydroxylase immunoreactive neurons nigral neurons (**D**) is preserved within the midbrain of MAM treated mice.

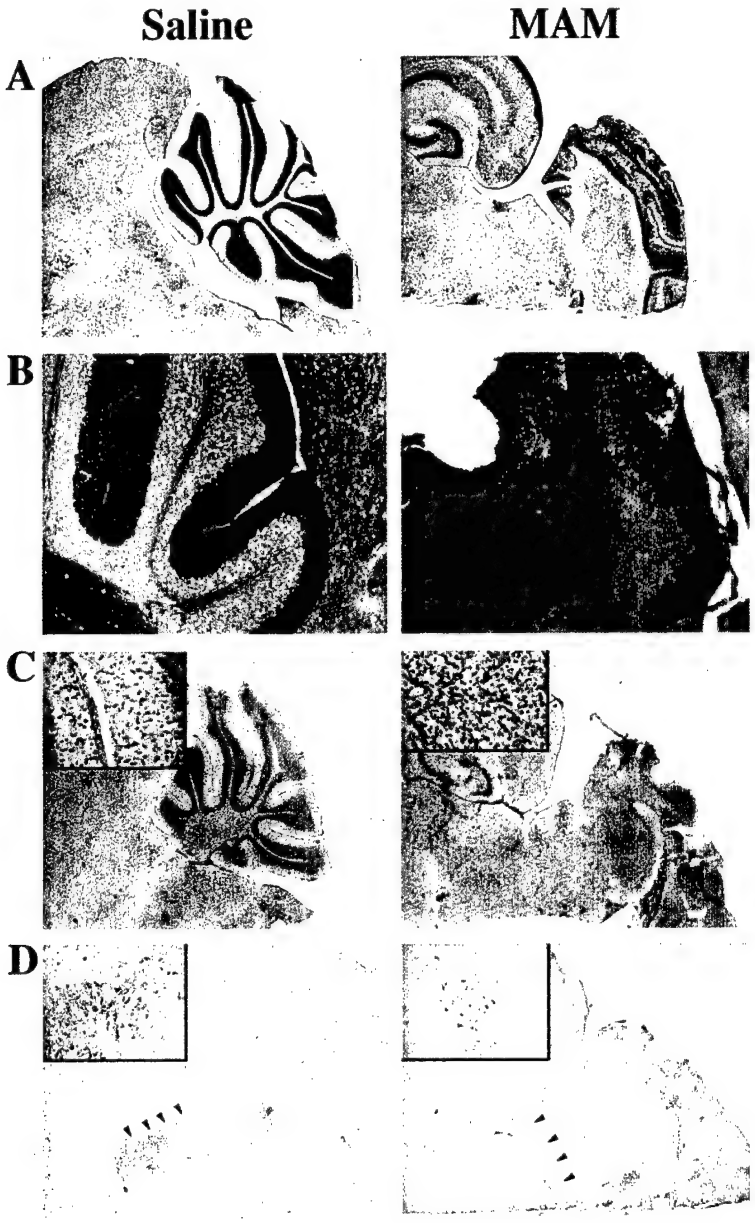

Figure 12. Neuropathology of the Brain from Postnatal MGMT$^{-/-}$ Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B,C**] or midbrain [**D**] of 22 day-old pups treated at postnatal day 3 with saline or MAM (21.5 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the smaller cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections. At the light microscopic level (**B & C**), smaller cerebellar folia, thinner cerebellar cortex and disorganization and degeneration of neurons in the granule, Purkinje and molecular layers were observed. CaBP was particularly useful for visualizing the disorganization and abnormal appearance of Purkinje cells within the cerebellum of MAM treated animals (**B**). Note also the reduced staining for tyrosine hydroxylase and the abnormal organization of nigral neurons (**D**) within the midbrain of MAM treated mice.

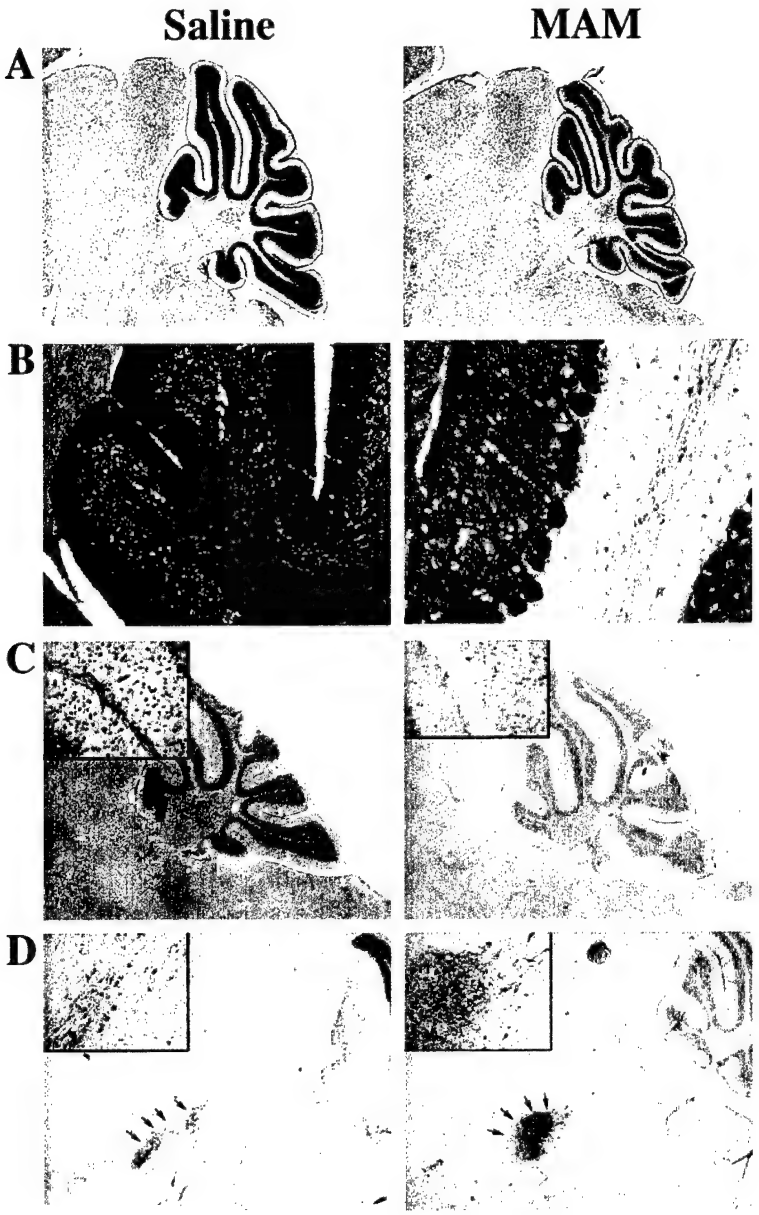

Figure 13. Neuropathology of the Brain from Postnatal MGMT^{+} Mice treated with MAM. Light micrographs of representative areas from sagittal sections (25 μm) of the cerebellum [**A,B,C**] or midbrain [**D**] of 22 day-old pups treated at postnatal day 3 with saline or MAM (43 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Alternate tissue sections were immunoprobed with antibodies to the calcium-binding protein calbindin (**B**) or to tyrosine hydroxylase to label dopaminergic cells (**D**). Note the mild atrophy of the cerebellum of MAM *vs.* saline treated mice (**A**) in cresyl violet stained sections when compared to moderately dosed MGMT$^{-/-}$ mice (*see* **Figure 12A**). At the light microscopic level (**B**), the cerebellar folia and the organization of neurons in the granule, Purkinje and molecular layers were preserved in CaBP (**B**) and silver stained (**C**) tissue sections of MAM treated mice. Note also the organization and staining intensity of tyrosine hydroxylase immunoreactive neurons nigral neurons (**D**) is preserved within the midbrain of MAM treated mice.

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DRAFT

DNA REPAIR PROTECTS NEURONS FROM GENOTOXIN-INDUCED CELL DEATH

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ABSTRACT

DNA repair plays a pivotal role in protecting the genome from damage by endogenous and environmental genotoxins and may also be important for protecting the brain from acute or chronic nervous tissue injury. The present studies examine the role of different cellular DNA repair mechanisms in protecting neurons from acute genotoxin-induced injury. Methylazoxymethanol (MAM), an etiological candidate of the prototypical neurodegenerative disorder western Pacific ALS/PDC, and the neurotoxic bifunctional aldehyde chloroacetaldehyde (CAA) were used as model environmental genotoxins. Since DNA damage produced by MAM and CAA is reportedly repaired by the base-excision repair (BER) or nucleotide excison repair (NER) pathways, neuronal and astrocyte cell cultures derived from the cerebellum of wild type (C57BL/6) mice or mice deficient in the BER enzyme 3-alkyladenine DNA glycosylase (AAG) or NER enzyme xeroderma pigmentosum A (XPA) were treated with 10-1000 μM MAM or 1.0–50 μM CAA for 24h and examined for cell viability and DNA damage. *Aag*$^{-/-}$ neurons were more sensitive to CAA (>20 μM) than comparably treated wild type neurons and the sensitivity correlated with the increased level of TUNEL^{+} cells. *Aag*$^{-/-}$ neurons were also equally sensitive to the alkylating agent 3-methyllexitropsin (Me-Lex), but insensitive to MAM. In contrast, cerebellar astrocytes from *Aag*$^{-/-}$ mice were relatively insensitive to both CAA and MAM. However, *Xpa*$^{-/-}$ neurons were more sensitive than wild type or *Aag*$^{-/-}$ neurons to MAM (>100 μM) and low concentrations of CAA (>10 μM). These studies demonstrate that DNA damage is an important underlying mechanism of MAM and CAA-induced neurotoxicity and that specific pathways of DNA repair are responsible for protecting neurons from insult by different types of DNA damaging agents. Consequently, the nervous system of individuals with compromised DNA repair may be particularly vulnerable to insult by environmental genotoxins.

Key Words: Alkyladenine DNA glycosylase (*Aag*), xeroderma pigmentosum group A (*Xpa*), cerebellar neurons, astrocytes, methylazoxymethanol (MAM), chloroacetaldehyde

Running Title: DNA repair protects neurons from genotoxin-induced cell death.

INTRODUCTION

DNA is constantly under attack by endogenous and environmental agents. It has been estimated that human (non-neuronal) cells lose approximately 10,000 purines and 200 pyrimidines from their genome per day as a result of normal oxidative metabolism (Mullaart et al. 1990;Richter et al. 1988). In addition, damaged bases and baseless sites are produced in DNA by the action of exposure to various environmental agents (e.g., chemotherapeutics, ionizing radiation, pesticides, metals) (Lindahl 1993). To overcome the harmful and possible irreversible effects of DNA damage, cells have at their disposal a repertoire of DNA-repair systems that are able to remove or circumvent lesions that might otherwise interfere with DNA replication and transcription or trigger cell death mechanisms (e.g., apoptosis). The base-excision (BER) and nucleotide excision (NER) DNA repair pathways are two key cellular pathways for recognizing and removing alkylated, bulky adducts, or oxidative DNA damage induced by environmental or endogenous (e.g., reactive oxygen species) agents (Huang et al. 1994;Reardon et al. 1997). As a chemical class, simple alkylating agents (e.g., methane methylsufonate, methylnitrosourea) produce over a dozen DNA lesions (Singer and Hang 1997) and most of these are substrates for enzymes of the BER pathway. N^7-Alkylpurines, N^3-alkylpurines and ethenobase DNA adducts are removed by the BER enzyme 3-methylpurine DNA glycosylase (MPG or AAG) through a mechanism involving cleavage of the glycosylic bond between the modified base and the deoxyribose sugar (Roy et al. 1998;Dosanjh et al. 1994;Memisoglu and Samson 2000b;Wyatt et al. 1999;Asaeda et al. 2000); the resulting abasic site is recognized by AP endonucleases that cleave the phosphodiester bond 5' to the abasic site to form a strand break. DNA polymerase β and a DNA ligase (I or III) restore the DNA template to the original sequence by inserting the correct nucleobase and ligating the DNA backbone, respectively. UV-irradiation and chemical agents (e.g., cisplatin, mitomycin C, 4-nitroquinoline, psoralens) produce bulky DNA adducts that are repaired by nucleotide excision repair (NER), a separate and complex cellular pathway that requires the coordination of at least 2 dozen different proteins (Wood 1997). Recognition of bulky DNA adducts by NER enzymes is considered the rate-limiting step and involves the

coordination of several proteins including xeroderma pigmentosum complementation group A protein (XPA) and replication protein A (RPA) complex and the xeroderma pigmentosum complentation group C protein (XPC) and human Rad23B protein (hH23B) complex (Batty and Wood 2000). NER can also function as a secondary cellular pathway for the repair of oxidative (Reardon et al. 1997) and alkylation-induced (Huang et al. 1994) DNA damage.

Neurons of the developing (Woodhead et al. 1985;Schmitz et al. 1999;Washington et al. 1989;Likhachev et al. 1983;Rao et al. 2000) or mature (Gaubatz and Tan 1994;Kleihues et al. 1980;Brooks et al. 1996;Cardozo-Pelaez et al. 2000;Ide et al. 2000;Buecheler and Kleihues 1977) CNS are reportedly inefficient at repairing DNA damage suggesting that these cells may be particularly vulnerable to damage by environmental genotoxic agents. Chemotherapeutic agents, solvents, metals or pesticides are a few examples of chemicals that are reported to damage brain tissue DNA and induce acute or delayed brain tissue injury (Levay et al. 1997;Mehl et al. 2000;McVay and Wood 1999;Stedeford et al. 2001;Renis et al. 1996;Anderson et al. 1996). The formation of DNA adducts by these compounds in brain cells or tissue has been suggested as evidence that DNA damage plays an important role in the neurotoxic properties of these agents (Brooks 2000;De Flora et al. 1996;Stedeford et al. 2001;Renis et al. 1996;Mehl et al. 2000). However, most of these agents also induce effects on other cellular processes that can lead to cell death by alternative mechanisms. Clearly, the current approaches have not provided conclusive evidence that DNA damage is an important mechanism underlying either acute or chronic brain tissue injury. The recent development of transgenic mouse mutants for a variety of DNA repair pathways provides an alternative approach for clarifying the role of DNA damage in brain tissue injury. We have taken this approach in the present studies by comparing the survival of cultured neurons from different DNA repair mutant mice after acute treatment with environmental genotoxic agents.

The present studies compare the well established DNA damaging properties of the developmental neurotoxin methylazoxymethanol (MAM) with the environmental genotoxin chloroacetaldehyde (CAA) to explore the mechanism by which genotoxins induce acute brain tissue injury. MAM is a widely used tool by neurobiologists to disturb the developing CNS of animals (Cattabeni and Di Luca 1997;Colacitti et al. 1999) and is a strong etiological candidate for a prototypical neurodegenerative disorder in the western Pacific with features of ALS, Parkinson's disease and an Alzheimer-like dementia (ALS/PDC) (Zhang et al. 1996;Spencer et al. 1991;Eizirik et al. 1996;Kisby et al. 1992). MAM also induces persistent DNA damage in neuronal cultures (Esclaire et al. 1999) or the brains of rodents treated *in utero* with the genotoxin (Kisby et al. 1999). The mechanism of MAM induced neuronal cell death is poorly understood, but the prevailing hypothesis is that it targets proliferating (Johnston and Coyle 1979), as well as post-mitotic neurons (Hoffman et al. 1996;Esclaire et al. 1999), by damaging DNA. Chloroacetaldehyde (CAA) is a chlorinatation by-product of finished drinking water supplies (Munter et al. 1996), a contaminant at hazardous waste sites (Park et al. 1993;Malaveille et al. 1975), and a toxic metabolite of a wide variety of industrial chemicals (e.g., vinyl chloride, urethane, dichloroethylene) (Purchase et al. 1987) and chemotherapeutic agents (e.g., cyclophosphamide, ifosfamide) (McVay and Wood 1999;Shaw et al. 1983;Borner et al. 2000) and, therefore, is a more pervasive environmental genotoxin than MAM. Individuals on ifosfamide chemotherapy experience mild somnolence and confusion to severe encephalopathy and coma (Sood and O'Brien 1996) at serum concentrations of 10 μM to 100 μM CAA, effects also observed after acute intoxication with vinyl chloride (Langauer-Lewowicka et al. 1983). Irreversible effects on the extrapyramidal system have also been observed following ifosfamide chemotherapy (Anderson and Tandon 1991). Individuals chronically exposed to vinyl chloride have been reported to develop sensory-motor polyneuropathy, trigeminal sensory neuropathy, slight pyramidal signs and cerebellar and extrapyramidal motor disorders (Podoll et al. 1990;Polakowska 1990;Langauer-Lewowicka et al. 1983;Ding et al. 1989). The neurotoxic properties of MAM are reportedly linked to the ability of this genotoxin to form alkylguanine

(e.g., N^7-methylguanine) DNA adducts (Matsumoto 1985;Matsumoto et al. 1972). The mechanism of CAA-induced neurotoxicity is less clear than MAM, but is proposed to involve oxidative stress (Sood and O'Brien 1996;McVay and Wood 1999) and/or the generation of ethenobase DNA adducts (Pallotta et al. 1992;Anderson and Tandon 1991;Shuper et al. 2000). While previous studies indicate that DNA damage induced by MAM or CAA can accumulate or persist within CNS or peripheral tissues (Ciroussel et al. 1990a;Esclaire et al. 1999;Kisby et al. 1999;Zielinski and Hergenhahn 2001), it is not clear if the DNA damage generated by these genotoxins is the primary mechanism of nervous tissue injury. To further explore the relationship between DNA damage and neurotoxicity, the cytotoxic properties of MAM and CAA were evaluated in neuronal and astrocyte cell cultures derived from wild-type mice and mice that are defective in base-excision (i.e., AAG) or nucleotide excision (i.e., XPA) DNA repair. These mouse mutants were chosen because these DNA repair proteins are reported to play a key role in removing the DNA damage generated by MAM (O'Connor and Laval 1991;Singer and Hang 1997;Asaeda et al. 2000) or CAA (Singer and Brent 1981;Singer and Hang 1997;Dosanjh et al. 1994). Results from these studies demonstrate for the first time that the BER and NER pathways play a vital role in protecting mature neurons from the cytotoxic effects of environmental genotoxins, possibly *via* a DNA damage mechanism.

METHODS

Mutant Mice

Alkyladenine DNA glycosylase (*Aag*$^{-/-}$) or xeroderma pigmentosum group A (*Xpa*$^{-/-}$) knock-out mice were generated in C57BL/6 mice by inserting a *neo* expression cassette into exon 2 of the mouse *Aag* gene (Engelward et al. 1997b) or by inserting a *neo* expression cassette into exon 4 of the mouse *Xpa* gene (Nakane et al. 1995) using ES cell techniques and the intercrossing of heterozygous (*Aag*$^{+/-}$ or *Xpa*$^{+/-}$) mice. *Aag*$^{-/-}$ or *Xpa*$^{-/-}$ mice were generated from intercrosses of *Aag* or *Xpa* heterozygous mice (+/-) and litters from the intercrossing of *Aag*$^{-/-}$ or *Xpa*$^{-/-}$ mice used to prepare neuronal and astrocyte cell cultures. The brains of 6-8 day old C57BL/6 (wild-type), *Aag*$^{-/-}$, or *Xpa*$^{-/-}$ mice were placed in Hibernate/B27 cell culture media (GibcoBRL) for the preparation of neuronal and astrocyte cell cultures.

Neuronal and Astrocyte Cell Cultures

Primary mouse granule cell or astrocyte cell cultures were prepared from the cerebella of 6-8-day old neonatal C57BL/6 (wild type), *Aag*$^{-/-}$, or *Xpa*$^{-/-}$ mice by placing the tissue in ice-cold Hibernate/B27 cell culture media (GibcoBRL) and dissociating the tissue in BSS with 0.1% trypsin as previously described by Kisby and Acosta (Weiss and Choi 1991) and Meira *et al.* (Meira et al. 2001). Cell cultures were prepared by diluting the cell pellet (*neurons*) or supernatant (*astrocytes*) with high potassium (25 mM) containing plating media (Neurobasal™ Media with 0.5 mM glutamine, 10% FCS, 10% HS, 2% B27 supplement) and seeded at a density of 0.1-0.2 x 10^6 or 0.01-0.02 x 10^6 cells/well of a 48-well plate coated with (*neurons*) or without (*astrocytes*) poly-D-lysine (BioCoat™, BD Biosciences). Neurons were allowed to attach for 2h while astrocytes were allowed to attach for 24h before replacing the culture media with neuronal

(Neurobasal™ media with 0.5 mM glutamine, 2% B27 supplement, 25 mM KCL) or astrocyte (DMEM with 10% FCS, 2.0 mM glutamine, 0.25 β-mercaptoethanol) maintenance media. Neuronal and astrocyte cell cultures were fed weekly by adding fresh culture media to the wells and the cells maintained for 7 days (*neurons*) or 10-14 days (*astrocytes*) before treatment with 10-1000 μM methylazoxymethanol (MAM), 0.1-50 μM chloroacetaldehyde (CAA) or 0.1-50 μM Me-Lex [$MeOSO_2(CH)_2$-*N*-methylpyrrole dipeptide], a well-established alkylating agent (Kelly et al. 1999) kindly provided by Dr. B. Gold (Univ. Nebrasksa).

TUNEL Labelling

Primary cerebellar neuronal cultures treated for 24h with MAM (10 μM, 100 μM, 1000 μM) or CAA (1.0 μM, 10 μM, 50 μM) were examined for DNA damage using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) with the NeuroTacs™ staining kit according to the manufacturer's instructions (Trevigen, Gaithersburg, MD). After toxin treatment, cells (on 8-well chamber slides) were fixed with 4% buffered paraformaldehyde and the incorporation of biotinylated nucleotides determined by incubating the cells with NovaRed™ (Vector Labs, Inc). Slides were washed, lightly counterstained with methyl green (Vector Labs, Inc.), mounted and the cells examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0). For quantitative studies, 5 random fields (~200-500 cells/field of cells with prominent nuclei, n= 3 slides) were counted by two different observers and the values expressed as the mean ± SEM of immunopositive cells ($TUNEL^+$). Interobserver variation was always less than 1%.

Cell Viability

Live/Dead Assay

Mouse neuronal and astrocyte cell cultures treated with control media or media supplemented with various concentrations of MAM or CAA were examined by fluorescence microscopy for cell viability using the fluorochromes calcein-AM and propidium iodide (PI) as previously described by Kisby *et al.* (Kisby et al. 2000) and Meira *et al.* (Meira et al. 2001). Briefly, the media over control, MAM or CAA-treated cultures was removed, replaced with control media containing 3.0 μM PI (a marker of cell damage) and 0.26 μM calcein-AM (a marker of cell viability), and the cultures treated for 10 min in a humidified 5%CO_2/O_2 incubator. The fluorochrome containing media was aspirated, the cultures washed once with control media, photomicrographs taken of the cell monolayer by epifluorescence microscopy, and cell survival examined on a fluorescence microplate reader (GeminiXS™, Molecular Devices) with well-scan capabilities. Values were expressed as the mean % surviving cells of controls ± SEM (n= 6/treatment group x 2-3 separate experiments).

Redox Function

Alamar blue™ (Trek Diagnostic Systems, Inc.) is a non-toxic metabolic indicator of viable cells that becomes fluorescent upon mitochondrial reduction and has been widely used to measure mitochondrial function in different cell systems (including neurons) (White et al. 1996;Springer et al. 1998). Mitochondrial function was determined in MAM and CAA treated neuronal or astrocyte cell cultures by adding Alamar Blue™ to a final concentration of 10% and the cells incubated at 37°C in a humidified 5%CO_2/O_2 incubator for 4h. Viability was measured when the medium in control wells turned blue to pink, typically at ~4h for both neurons and astrocytes.

Alamar blue™ fluorescence was measured in a FLUOstar™ (BMG LabTechnologies) automated plate-reading fluorometer, with excitation at 530 nm and emission at 590 nm. Values are reported as % redox activity of controls (n= 6/treatment group x 2-3 separate experiments).

Statistical Analysis

Data are expressed as the mean ± S.E.M. All data obtained were evaluated for statistical significance by one-way analysis of variance (ANOVA) and Scheffe's method of comparison. A probability value of $p < 0.05$ was considered significant unless otherwise noted.

RESULTS

DNA Damage Studies

Previous studies indicate that MAM induces DNA damage (i.e., N^7-methylguanine DNA adducts and O^6-methylguanine DNA adducts) in mature rat cortical neuronal cultures (Esclaire et al. 1999) while DNA damage (i.e., ethenobase DNA adducts) induced by the metabolites chloroacetaldehyde (CAA) or chloroethylene oxide is elevated in the brains of newborn rats following inhalation of vinyl chloride (Ciroussel et al. 1990b). Inefficient removal of DNA damage induced by MAM or the metabolite CAA can lead to strand breaks and ultimately cell death. To determine if DNA damage (i.e., strand breaks) is a characteristic feature of these genotoxins, neuronal cultures prepared from C57BL/6 (wild-type) mice were treated with various concentrations of MAM or CAA and the extent of TUNEL labeling measured (**Figure 1**). TUNEL labeling of neurons increased with the concentration of either genotoxin, but significant levels (50-60%) were only observed at high concentrations of MAM (1000 μM) or CAA (10 μM and 50 μM). These studies demonstrate that DNA damage is a characteristic feature of mature post-mitotic neurons treated with the genotoxins MAM and CAA.

Viability of DNA Repair Deficient Neurons

We have shown that MAM and CAA induce neuronal DNA damage, but whether this mechanism is responsible for their neurotoxic properties is not known. Since DNA damage induced by MAM and CAA is reportedly repaired by either the BER or NER pathways (Hang et al. 1996;Dosanjh et al. 1994;Elder et al. 1998), neurons that are deficient in these repair pathways would be expected to be more sensitive to these genotoxins. This hypothesis was tested by examining the cytotoxic properties of MAM and CAA in cerebellar neuronal cultures

derived from mice deficient in BER (i.e., 3-alkylguanine DNA glycosylase, $AAG^{-/-}$) or NER (i.e., xeroderma pigmentosum group A, $XPA^{-/-}$). Mature neuronal cultures prepared from the cerebellum of 7-day old wild type, $AAG^{-/-}$ or $XPA^{-/-}$ mice were treated for 24h with different concentrations of MAM (**Figure 2**) or CAA (**Figure 3**) and examined for cell survival and mitochondrial function. In general, the viability of wild type cerebellar neurons declined with increasing concentration of either genotoxin. However, the survival of cerebellar neurons from the two DNA repair mutant mice differed following treatment with MAM or CAA. $AAG^{-/-}$ neurons were insensitive to MAM at concentrations >500 μM (*$p < 0.01$) (**Figure 2, top**), but very sensitive to CAA (50 μM) (**Figure 3**) when compared with similarly treated neuronal cultures from wild type mice. Redox activity, a measure of mitochondrial function (White et al. 1996), was significantly lower ($p < 0.01$) in $AAG^{-/-}$ neurons treated with 20 μM and 50 μM CAA than comparably treated wild type neurons or $AAG^{-/-}$ neurons treated with MAM. The lack of effect of MAM on mitochondrial function is consistent with the inability of this genotoxin to alter cytochrome oxidase activity in the cerebral cortex of rats (Ashwell and Webster 1987). In contrast, the survival of $XPA^{-/-}$ neurons was significantly reduced at 100 μM to 2000 μM MAM (**Figure 2**) or at lower concentrations (> 5 μM) of CAA (**Figure 3**). However, mitochondrial function of $XPA^{-/-}$ neurons was not influenced by MAM, but was severely reduced by CAA, suggesting that these two genotoxic agents differ in their mechanism of neurotoxicity. Skin fibroblasts and kidney epithelial cells derived from wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice also exhibited a similar pattern of sensitivity to CAA and MAM (GK, *unpublished data*) suggesting a common mechanism of toxicity across tissues. The increased sensitivity of $XPA^{-/-}$ neurons to MAM was unexpected since this genotoxin is a well known methylating agent (Esclaire et al. 1999) that produces DNA adducts (e.g., O^6-methylguanine, N^7-methylguanine) not known to be

substrates for NER. In comparison, $AAG^{-/-}$ and $XPA^{-/-}$ neurons were both sensitive to CAA, which is consistent with the ability of this genotoxin to generate ethenobase DNA adducts, a substrate for the BER (Borys and Kusmierek 1998;Dosanjh et al. 1994;Matijasevic et al. 1992) and NER (Ballering et al. 1997) pathways. The increased sensitivity of $AAG^{-/-}$ and $XPA^{-/-}$ skin fibroblasts and kidney epithelial cells to CAA (GK, *unpublished data*) is also consistent with this hypothesis.

Viability of DNA Repair Deficient Astrocytes

The above studies indicate that cerebellar neurons of $AAG^{-/-}$ and $XPA^{-/-}$ mice exhibit a differential sensitivity to the genotoxins MAM and CAA. Additional studies were conducted to determine if other CNS cell types also share this same sensitivity. For these studies, companion cultures of astrocytes (*from the cerebella of the same set of mice*) were treated in a similar fashion with MAM (**Figure 4**) or CAA (**Figure 5**) and examined for cell survival and mitochondrial function. Like neurons, the viability of astrocyte cell cultures from all mice generally declined with increasing concentration of either genotoxin. However, astrocyte cultures from wild type or $AAG^{-/-}$ mice were equally sensitive to CAA (**Figure 5**) and ~2.5x less sensitive ($p < 0.01$) than $AAG^{-/-}$ neurons treated with 10 μM or 50 μM CAA. These results suggest that among CNS cell types, neurons would be more vulnerable to CAA-induced toxicity. These findings are consistent with the reported neurotoxic side effects of ifosfamide at comparable serum concentrations (10-100 μM) of CAA (Sood and O'Brien 1996).

In parallel studies, companion astrocyte cell cultures from the cerebellum of wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice were treated with MAM and examined for viability. Like CAA treated

astrocyte cultures, there was a concentration-dependent decline in cell survival and mitochondrial function (**Figure 4**) after MAM treatment of astrocytes. Moreover, astrocyte cultures derived from all three DNA repair mutant mice were equally sensitive to MAM. Taken together, these results suggest that neurons are more vulnerable than astrocytes to insult by either CAA or MAM.

$AAG^{-/-}$ Neurons and DNA Damaging Agents

The above studies demonstrate that $AAG^{-/-}$ and $XPA^{-/-}$ neurons are especially sensitive to CAA, but insensitive to the alkylating agent MAM. The increased sensitivity of $AAG^{-/-}$ neurons to CAA may be due to the formation of ethenobase DNA adducts because these types of DNA adducts are substrates for mammalian 3-methyladenine DNA glycosylase (AAG) (Matijasevic et al. 1992;Engelward et al. 1997b) and possibly NER (Shah et al. 2001). In contrast, MAM is not known to form 3-methyladenine DNA adducts, which is consistent with the observed insensitivity of $AAG^{-/-}$ neurons to MAM. The role of DNA damage in CAA-induced neurotoxicity was explored further by examining the sensitivity of $AAG^{-/-}$ and $XPA^{-/-}$ cerebellar neuronal cell cultures to another genotoxin $MeOSO_2$ $(CH_2)_2$-lexitropsin (Me-Lex). Me-Lex is a synthetic analogue of the alkylating agent dimethyl sulfate that produces predominantly (>90%) 3-methyladenine DNA adducts (Kelly et al. 1999). The formation of these DNA adducts is reported to be responsible for the increased sensitivity of AAG deficient mammalian or bacterial cells to Me-Lex (Chen et al. 1994;Engleward et al. 1996). If the pattern of sensitivity of $AAG^{-/-}$ and $XPA^{-/-}$ cerebellar neurons is similar for Me-Lex and CAA, it would suggest that the neurotoxic effects of these genotoxins are related to DNA damage. To test this hypothesis, cerebellar neuronal cultures from wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice were treated with Me-Lex

for 24h and examined for cell survival and mitochondrial function (**Figure 6**). Like $AAG^{-/-}$ cerebellar neurons treated with CAA, $AAG^{-/-}$ neurons were very sensitive at high concentrations of Me-Lex (50 μM) (**Figure 6**) when compared with similarly treated neuronal cultures from wild type or $XPA^{-/-}$ mice. This sensitivity may be due to the formation of m^3-adenine DNA adducts because ~27% (± 3.2) and 100% (± 0.5) $TUNEL^+$ wild type neurons were observed after treatment with 10 μM or 50 μM Me-Lex, respectively. Comparable levels of DNA damage were also noted after treatment of wild type cerebellar neurons with 10 μM Me-Lex or 10 μM CAA (*compare with* **Figure 1**). However, DNA damage was more extensive after treatment with 50 μM Me-Lex than at equivalent concentrations of CAA suggesting m^3-adenine DNA adducts are more lethal to neurons than ethenobase DNA adducts. These studies demonstrate that $AAG^{-/-}$ and $XPA^{-/-}$ neurons are sensitive to genotoxins that generate 3-methyladenine and ethenobase DNA adducts, but not N^7-methylguanine or O^6-methylguanine DNA adducts. Consequently, 3mAde and ethenobase DNA adducts may be responsible for the neurotoxic properties of these DNA damaging agents.

Brain Tissue Levels of AAG

The BER enzyme alkyladenine DNA glycosylase (AAG) has been reported to remove ethenobase DNA adducts generated by CAA (Dosanjh et al. 1994;Engelward et al. 1997a) or m^3-adenine DNA adducts generated by Me-Lex (Engelward et al. 1997b;Monti et al. 2002). The above studies demonstrate that AAG deficient neurons are more vulnerable to the unrelated genotoxins CAA or Me-Lex than the simple methylating agent MAM. Therefore, the regional distribution of AAG could be an important factor that determines the vulnerability of neurons to different genotoxins. Protein extracts were prepared from the cortex, hippocampus, and

substantia nigra of a 51 yr old male subject (*non-neurological control*) and examined by western blotting (**Figure 7**) to determine if there are regional differences in the distribution of AAG. AAG was detected in human brain tissue (~ 32 kDa) with multiple bands occurred within some regions suggesting that the antibody recognized several isoforms or cross-reacted with other tissue DNA glycosylases. More importantly, AAG levels varied among brain regions with high levels detected in the substantia nigra and ~2x lower levels in the cortex. The heterogenous distribution and expression of AAG within the human brain suggests that certain regions would be more efficient than others at repairing DNA damage induced by environmental genotoxins (e.g., CAA).

DISCUSSION

There is compelling evidence that DNA damage plays an important role in the death of neurons during CNS development (Martin 2001) and in neurodegenerative disease (Rolig and McKinnon 2000). Reactive oxygen species and lipid peroxides are examples of endogenous sources that are reportedly responsible for the elevated levels of DNA damage observed in the brains of subjects with stroke and chronic neurodegenerative disease (e.g., ALS, Parkinson's disease, Alzheimer's disease) (Chopp et al. 1996;Sun and Cheng 1999;Rolig and McKinnon 2000). In addition, millions of individuals in the US are exposed each year to environmental agents that are both neurotoxic and capable of inducing DNA damage and some examples are alkylating antineoplastic agents, metals, and pesticides (Keshava and Ong 1999;Povey 2000). A key, but often overlooked characteristic of the DNA damage induced either by endogenous sources or environmental agents is that they form multiple types of DNA lesions (Lindahl 1993). For example, reactive oxygen species are reported to generate at least 20-30 different types of DNA adducts (Dizdaroglu 1992) while simple alkylating agents react with DNA to form N^7-alkyl-, N^3-alkyl- and O^6-alkyl purines (Singer 1979;Singer and Hang 1997). In addition, the abundance and spectrum of these DNA adducts can vary considerably among tissues and from one agent to another. These biochemical properties of DNA damaging agents have made it difficult to determine if a specific DNA adduct is responsible for the cytotoxic or neurotoxic properties of a particular endogenous or environmental agent. In the majority of studies, the abundance and type of DNA lesions produced in diseased brain tissue (Alam et al. 1997;Lyras et al. 1997;Alam et al. 2000) or following exposure to a genotoxic agent (Cabelof et al. 2002;Stedeford et al. 2001;Valverde et al. 2002;Bagchi et al. 2001;Geller et al. 2001) has been taken as evidence of

the link between DNA damage and brain tissue injury. Although these studies have established associations between DNA damage and brain injury, they do not directly determine if DNA damage was a primary (or secondary) event. The recent development of transgenic and knock-out models for a variety of DNA repair genes provides a novel approach for (i) identifying critical primary DNA lesions out of broad spectrum of induced DNA damage, (ii) modifying neuronal and/or brain tissue sensitivity to a particular genotoxin, and (iii) assessing the contribution of particular DNA repair proteins and repair pathways that protect neurons from DNA damage (Kaina et al. 1998). This approach was used in the present studies to begin unraveling the importance of DNA damage in brain tissue injury.

Our primary focus was to examine the relationship between DNA damage and acute brain injury by comparing the viability of neuronal cultures derived from DNA repair mutant mice after exposure to agents that induced different types of DNA damage. The long-term effects of these genotoxins on neuronal cultures from DNA repair mutant mice will be reported separately. For these studies, we examined the impact of two model genotoxins, the alkylating agent MAM and the bifunctional aldehyde chloroacetaldehyde, on the viability of neurons and astrocytes from the cerebellum of mice deficient in base-excision (i.e., alkyladenine DNA glycosylase, AAG) or nucleotide excision repair (i.e., xeroderma pigmentosum group A, XPA). The BER protein AAG or m^3A-DNA glycosylase has a wide substrate range with the ability to excise at least 17 different unrelated modified bases (Singer and Hang 1997) suggesting that this cellular protein may play an important role in protecting neurons from a wide variety of genotoxic agents. Among the modified bases repaired by AAG are N^7-methylguanine, ethenobases (e.g., ethenoadenine, ethenoguanine), and m^3-adenine, the predominant DNA adducts formed by the

genotoxins MAM (Nagata and Matsumoto 1969), CAA (Dosanjh et al. 1994), and Me-Lex (Kelly et al. 1999), respectively. Neuronal and astrocyte cell cultures from the cerebella of DNA repair mutant mice were used to compare the sensitivity of different CNS cell types to the genotoxins MAM, CAA and Me-Lex.

One of the characteristic features of the developmental genotoxin cycasin (and its aglycone MAM) is that it damages DNA (Nagata and Matsumoto 1969;Matsumoto et al. 1972;Matsumoto and Higa 1966;Esclaire et al. 1999;Mehl et al. 2000), an event that is reportedly linked with changes in gene expression and perturbations of brain neuronal mitosis and migration (Esclaire et al. 1999;Colacitti et al. 1999). These provocative observations suggest that DNA alkylation induced by MAM is responsible for the developmental and chronic effects of this genotoxin. Previous studies suggested that the cytotoxic properties of MAM are due to the formation of N^7-methylguanine DNA adducts (~70% of all DNA damage) in proliferating (Nagata and Matsumoto 1969;Matsumoto et al. 1972;Johnston and Coyle 1979) or post-mitotic neurons (Hoffman et al. 1996;Esclaire et al. 1999). However, N^7-methylguanine DNA adducts are known to be less toxic or mutagenic to cells than other methylated DNA bases (e.g., O^6-methylguanine)(Wild 1990;Walker et al. 2000), but they can spontaneously depurinate with age (Park and Ames 1988) to form potentially mutagenic abasic sites (Gentil et al. 1992;Neto et al. 1992). The present findings support this hypothesis by demonstrating that neurons deficient in AAG, a BER protein that repairs N^7-methylguanine DNA adducts (Asaeda et al. 2000), were relatively resistant to MAM-induced cell death. In addition, fibroblasts and other cells from AAG deficient mice are also insensitive to related simple methylating agents (e.g., mustards, nitrosoureas) that generate predominantly N^7-methylguanine DNA adducts, (Roth and Samson

2002;Powers et al. 2002). Therefore, it is unlikely that N^7-methylguanine DNA adducts are responsible for the severe loss of neurons that occurs in the developing fetus (e.g., cortex) or neonatal rat (e.g., cerebellum) 72 h after an *in utero* or postnatal injection of MAM, respectively (Sullivan-Jones et al. 1994;Cattabeni and Di Luca 1997). Rather, the increased sensitivity of cerebellar neurons, skin fibroblasts and kidney epithelial cells from O^6-methylguanine methyltransferase ($MGMT^{-/-}$) mice to MAM (Kisby et al. 2002;Kisby et al. 2001) suggests that O^6-methylguanine, although a minor DNA adduct (~1-6% of all MAM-induced DNA damage), is more likely responsible for the acute neurotoxic properties of MAM. The unexpected increased resistance of $AAG^{-/-}$ neurons and other cell types to MAM is likely a protective mechanism that leaves these DNA adducts unrepaired because their repair would create more lethal lesions (i.e., abasic sites)(Turker et al. 1999).

The increased sensitivity of $XPA^{-/-}$ neurons to MAM was also an unexpected finding because the DNA adducts generated by this genotoxin (i.e., N^7-methylguanine, O^6-methylguanine and 8-methylguanine) (*reviewed in* (Kisby et al. 1999)) are not known to be primary substrates for nucleotide excision repair (NER) (Huang et al. 1994). As peviously stated, N^7-methylguanine is reportedly repaired by m^3-adenine DNA glycosylase (AAG or MPG) (Asaeda et al. 2000) or possibly another DNA glyosylase (Smith and Engelward 2000), O^6-methylguanine is repaired by O^6-methylguanine methyltransferase (MGMT) (Pegg et al. 1995) and the repair of 8-methylguanine DNA adducts is unknown. However, studies in bacteria, yeast and mammalian cells suggest that there is considerable cross-talk between NER and other DNA repair pathways (e.g., BER, MGMT) (Huang et al. 1994;Memisoglu and Samson 2000b), which may explain the increased sensitivity of $XPA^{-/-}$ neurons to MAM. In *E. coli* and yeast, NER-deficient mutants

have been shown to be sensitive to alkylating agents that generate N^7-methylguanine, m^3-adenine or O^6-methylguanine DNA adducts (Memisoglu and Samson 2000a;Samson et al. 1988;Samson et al. 1997). More recently, the alkylating agent *N*-n-butyl-*N*-nitrosourea (BNU) was found to be 2-3x more mutagenic in the *hprt* locus of $XPA^{-/-}$ mouse splenocytes than from normal mice after a single i.p. injection (Bol et al. 1999). BNU was also 2.7x more toxic to $XPA^{-/-}$ fibroblasts than similarly treated cells derived from wild type mice. These studies suggest that NER and AGT are required by cells to protect them from the both the mutagenic and cytotoxic effects of alkylating agents. Given the increased sensitivity of $MGMT^{-/-}$ neurons, fibroblasts and kidney epithelial cells (Kisby et al. 2002;Kisby et al. 2001) and $XPA^{-/-}$ neurons (*present studies*) to MAM, a similar mechanism may operate within neurons to protect them from the cytotoxic effects of alkylating agents. Findings from these studies also demonstrate that multiple DNA repair pathways are required to protect neurons from insult by MAM. Whether insult by other environmental genotoxic agents also proceeds by a similar or different neuronal mechanism(s) requires further study to determine the importance of these processes in protecting the brain from acute or chronic tissue injury.

Another objective was to determine if specific neuronal DNA repair pathways are responsible for protecting neurons from the formation of different types of DNA adducts induced by environmental genotoxins. For these studies, we compared the viability of $AAG^{-/-}$ and $XPA^{-/-}$ neurons after treatment with MAM, CAA, or Me-Lex, each genotoxin producing a different DNA adduct (i.e., N^7-methylguanine, ethenobase or 3-methyladenine DNA adducts, respectively). Chloroacetaldehyde (CAA) is a genotoxic metabolite of industrial chemicals (e.g, vinyl chloride, dichloroethylene, urethane) and antineoplastic agents (e.g., ifosfamide,

cyclophosphamide) that reacts with DNA to form the exocyclic DNA adducts ethenoadenine (εA), ethenocytosine (εC) and ethenoguanine (eG)(Singer and Hang 1999;Oesch et al. 1986). These ethenobase DNA adducts are also formed endogenously *via* the reaction of lipid peroxides (e.g., 4-hydroxynonenal) with DNA (Nair et al. 1999). Me-Lex, a methyl sulfonate ester of a neutral *N*-methylpyrrolecarboxamide-based dipeptide binds in the minor groove of DNA at AT sequences resulting in the predominant methylation (>90%) of the N3 position of adenine (m^3Ade) (Kelly et al. 1999). In marked contrast to CAA and Me-Lex, the methylation pattern of MAM results in methylation of nitrogen bases within the major groove of DNA to preferentially (~70%) generate N^7-methylguanine adducts (Matsumoto 1985). $AAG^{-/-}$ neurons exhibited comparable sensitivity towards both CAA and Me-Lex, but were insensitive to MAM while $XPA^{-/-}$ neurons were sensitive to all three genotoxic agents. These studies suggest that $AAG^{-/-}$ neurons are especially sensitive to genotoxins that generate m^3-Ade and ethenobases DNA adducts, but not agents that preferentially induce N^7-methylguanine DNA adducts. The increased sensitivity of $XPA^{-/-}$ neurons to all three genotoxins suggests that this DNA repair pathway provides a critical back-up repair system to other DNA repair proteins (e.g., AAG, AGT) that may be saturated after exposure to high concentrations of genotoxic agents. These findings also suggest that neurons possess specific DNA repair pathways for dealing with the wide spectrum of DNA adducts that may be formed by endogenous or environmental genotoxins. Consequently, factors that influence these neuronal DNA repair pathways may play an important role in altering the susceptibility of nervous tissue to either endogenous or exogenous genotoxins.

The selective vulnerability of neurons is a characteristic feature of ischemic or neurodegenerative

brain tissue or brain tissue injury following exposure to genotoxic agents. If DNA damage is an important mechanism of neuronal cell death in acute or chronic tissue injury, then neurons should be more vulnerable to DNA damage than other CNS cell types. Comparable studies were conducted with astrocytes and neurons from the same cerebellar tissue of DNA repair mutant mice to determine if neurons are preferential targets for environmental genotoxins. We show that cerebellar astrocytes, unlike neurons, from BER or NER deficient mice were equally sensitive to the genotoxins CAA and MAM. Therefore, astrocytes appear insensitive to these genotoxins by a mechanism of cell killing that differs from neurons or they are more proficient at repairing DNA damage than other CNS cell types. While there is little evidence for the former, the latter hypothesis is supported by recent studies demonstrating that astrocytes appear more resistant to simple alkylating agents than either oligodendrocytes or microglia (LeDoux et al. 1996;Ledoux et al. 1998). Viability studies demonstrated an increased sensitivity of oligodendroglia and microglia to *N*-nitrosoureas when compared to similarly treated astrocytes (LeDoux et al. 1996). Thus it appears that repair of O^6-methylguanine and *N*-methylpurine DNA adducts and possibly ethenobase DNA adducts is more proficient in astrocytes than in the other two glial cell types or neurons (*present studies*).

In summary, these studies suggest that neurons possess specific DNA repair pathways for dealing with the daily insult of damage to its genome by endogenous sources or environmental agents. Consequently, genetic or acquired factors could be important contributing factors that influence these cellular DNA repair pathways and alter the susceptibility of neurons to insult by endogenous or environmental genotoxic agents. The recent identification of polymorphisms for various DNA repair genes (e.g., MGMT, AAG) in the general human population (Ford et al.

2000;Shen et al. 1998;Hu et al. 2001;Hadi et al. 2000) suggests that the DNA repair machinery be compromised in certain individuals making them particularly susceptible to the neurotoxic and possibly neuro-oncogenic properties of various environmental genotoxins. Recent studies indicate that aging (Rao et al. 2000;Kohama S.G. et al. 2000), diet (Prolla and Mattson 2001) or other lifestyle factors may also influence brain tissue DNA repair and, therefore, alter the vulnerability of the brain to either endogenous or exogenous DNA damaging agents. A better understanding of the cellular mechanisms that activate neuronal DNA repair may be an important area of future research for understanding how DNA damage induces acute or chronic brain tissue injury.

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Figure Legends

Figure 1. In situ DNA damage of wild type (C57BL/6) neurons by methylazoxymethanol (MAM) and chloroacetaldehyde (CAA). Representative light micrographs of primary mouse cerebellar neurons that were treated for 24h with various concentrations of MAM (10 μM-1000 μM) or CAA (1 μM-50 μM) and the fixed cells examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.), which detects DNA strand breaks by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique. The slides were washed, counterstained with methyl green (Vector Labs, Inc.), mounted and the cells examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0). For quantitative studies, 5 random fields (~200 cells/field of cells with prominent nuclei) were counted and the values expressed as the mean ± SEM of immunopositive cells (TUNEL+). Note the extensive TUNEL labelling of neurons treated with 50 μM CAA while higher concentrations of the alkylating agent MAM were required to obtain a similar amount of DNA damage. Significantly different from control *(*$p < 0.01$)*.

Figure 2. Viability of cerebellar neurons from wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice treated with methylazoxymethanol (MAM). Mouse cerebellar granule cell cultures were treated with 10 μM to 2000 μM MAM for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence (bottom). After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and the fluorescence measured by a microplate reader (top). Values represent the mean ± SEM (n=6, 2-3 separate

experiments). Significantly different from MAM treated wild-type cells (* $p < 0.05$, Δ $p < 0.01$).

Figure 3. Viability of cerebellar neurons from wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice treated with chloroacetaldehyde (CAA). Mouse cerebellar granule cell cultures were treated with 1.0 μM to 50 μM CAA for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and the cultures examined on a fluorescence microplate reader. Values represent the mean ± SEM (n= 6, 2-3 separate experiments). Significantly different from CAA treated wild-type cells (* $p < 0.05$, Δ $p < 0.01$, ** $p < 0.001$).

Figure 4. Viability of cerebellar astrocytes from wild type, $AAG^{-/-}$ and $XPA^{-/-}$ mice treated with MAM. Mouse cerebellar astrocyte cell cultures were treated with 10 μM to 1000 μM MAM for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and the cultures examined on a scanning fluorescence microplate reader. Values represent the mean ± SEM (n= 4, 2 separate experiments).

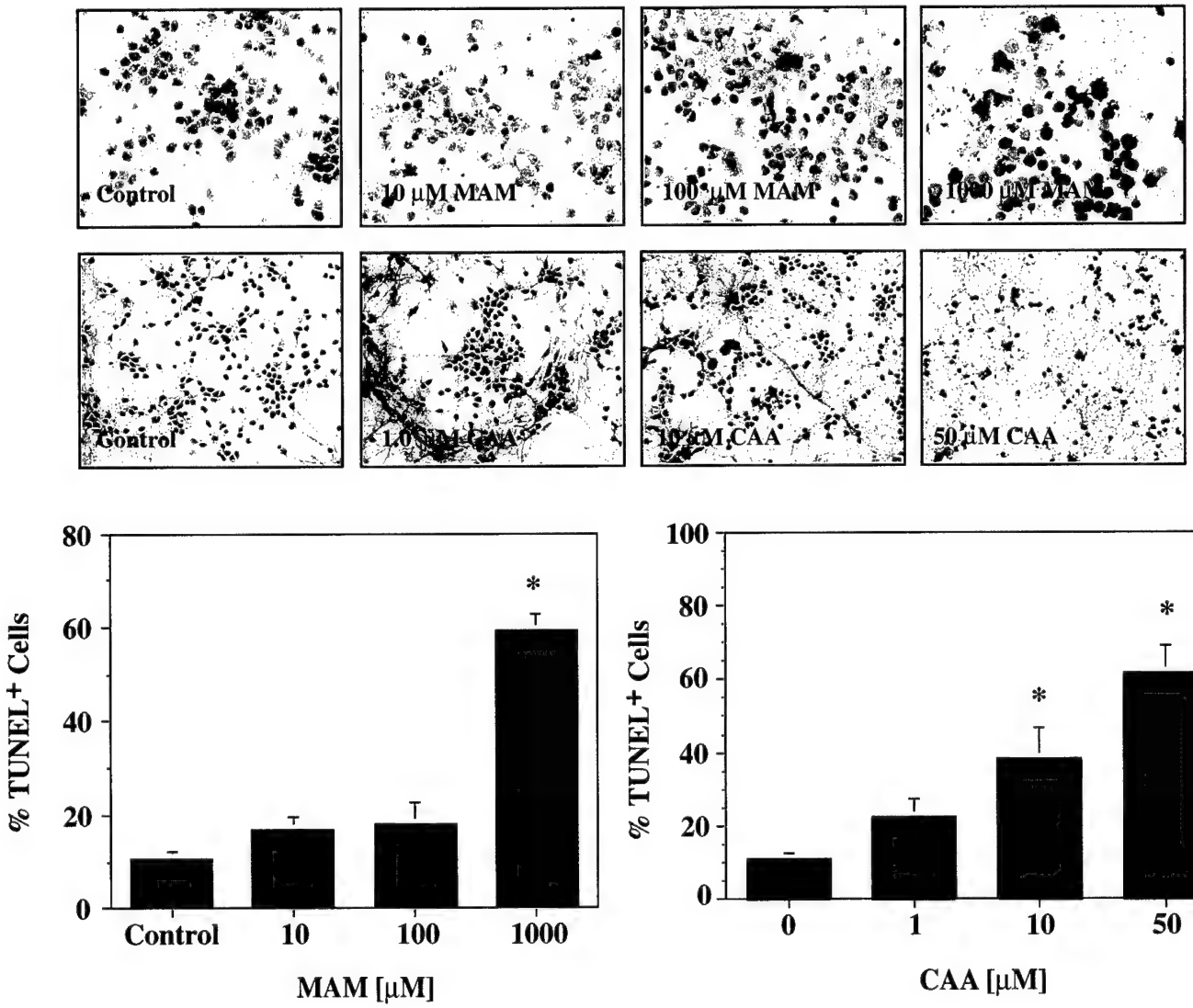
Figure 5. Viability of cerebellar astrocytes from wild type and $AAG^{-/-}$ mice treated with CAA. Mouse cerebellar astrocyte cell cultures were treated with 1.0 μM to 50 μM CAA for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide) and the cultures examined on a scanning fluorescence microplate

reader. Values represent the mean ± SEM (n= 4, 2 separate experiments).

Figure 6. Viability of cerebellar neurons from wild type, AAG-/- and XPA-/- mice treated with the alkylating agent methyl-lexitropsin (Me-Lex). Mouse cerebellar granule cell cultures were treated with 1.0 μM to 50 μM Me-Lex for 24h, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. After 4h, the cultures were incubated with 0.26 μM calcein-AM and 3.0 μM propidium iodide and the fluorescence measured on a scanning microplate reader. Values represent the mean ± SEM (n=6, 2 separate experiments). Significantly different from MAM treated wild-type cells (*$p < 0.05$, **$p < 0.01$).

Figure 7. *N*-Alkyladenine DNA glycosylase (AAG) levels in human brain tissue. A tissue homogenate was prepared from the brain of a 51-yr old male subject and an aliquot analyzed by western blotting for AAG levels. Protein extracts (30 μg) from various brain regions [frontal cortex, CTX; hippocampus, HP; substantia nigra, SN] and human recombinant MPG (hMPG) were electrophoresed on a 12%, 0.75 mm SDS PAGE-gel and the membrane probed with a monoclonal antibody to human MPG (1:2000). Specific binding of the primary antibody to MPG was visualized using a HRP-conjugated goat anti-mouse antibody (1:1000) and Enhanced Chemiluminescence (ECL™) (Amersham) according to the manufacturer's protocols. Bands were detected and quantified using a GS-363 phosphorimaging system (Molecular Analyst™, BioRad) as previously described (Kisby et al. 1997).

Figure 1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1.0 µM CAA
10 µM CAA
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Control
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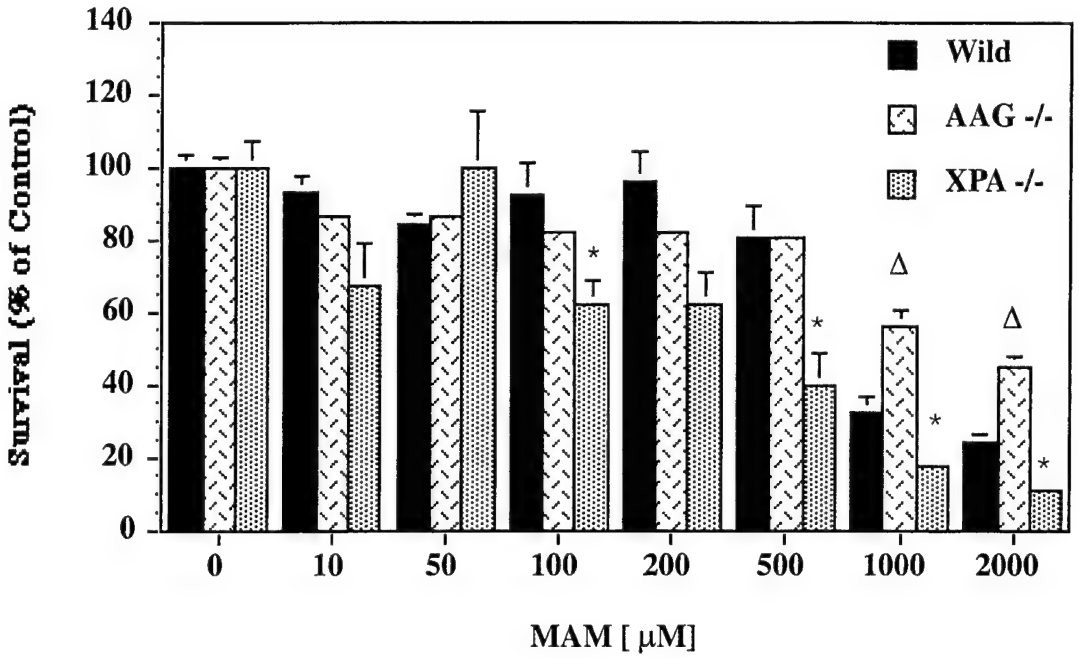
Figure 2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XPA -/-
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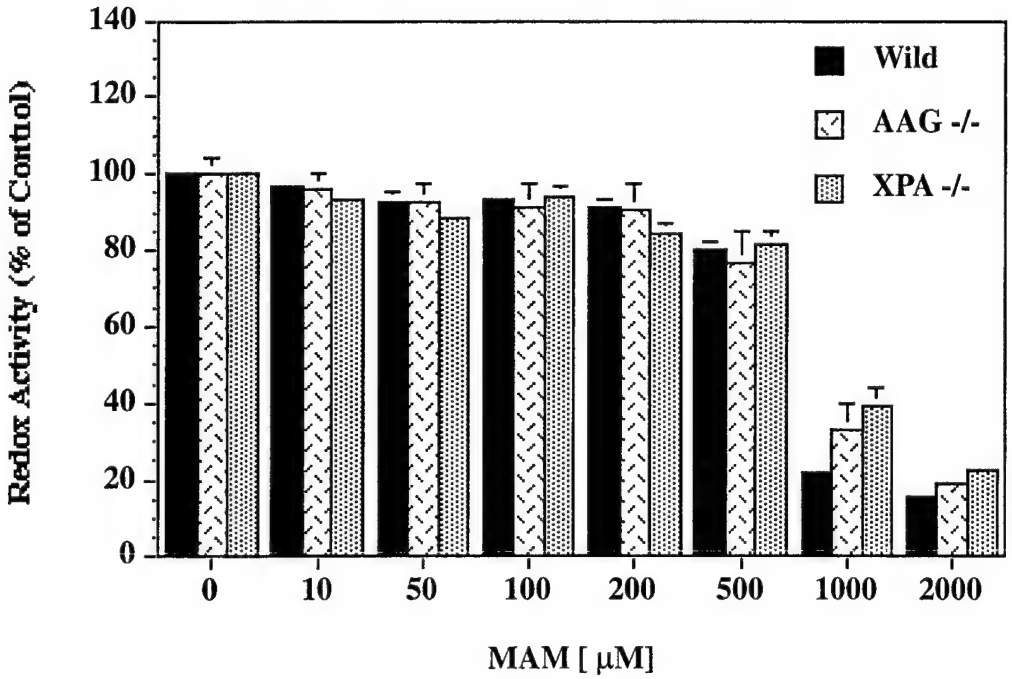
MAM [μM]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XPA -/-
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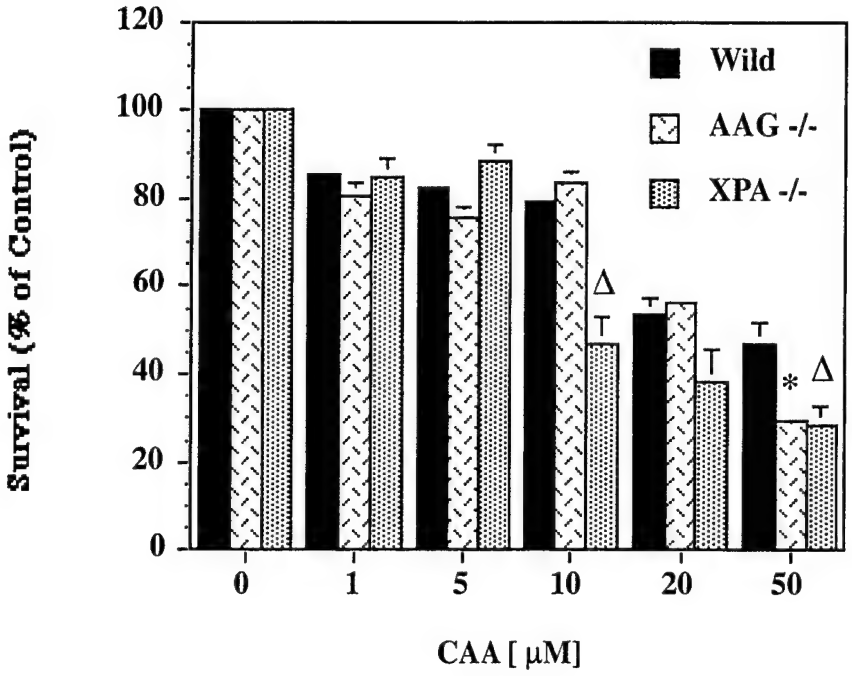
2000
MAM [μM]

Figure 3


Survival (% of Control)
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XPA -/-
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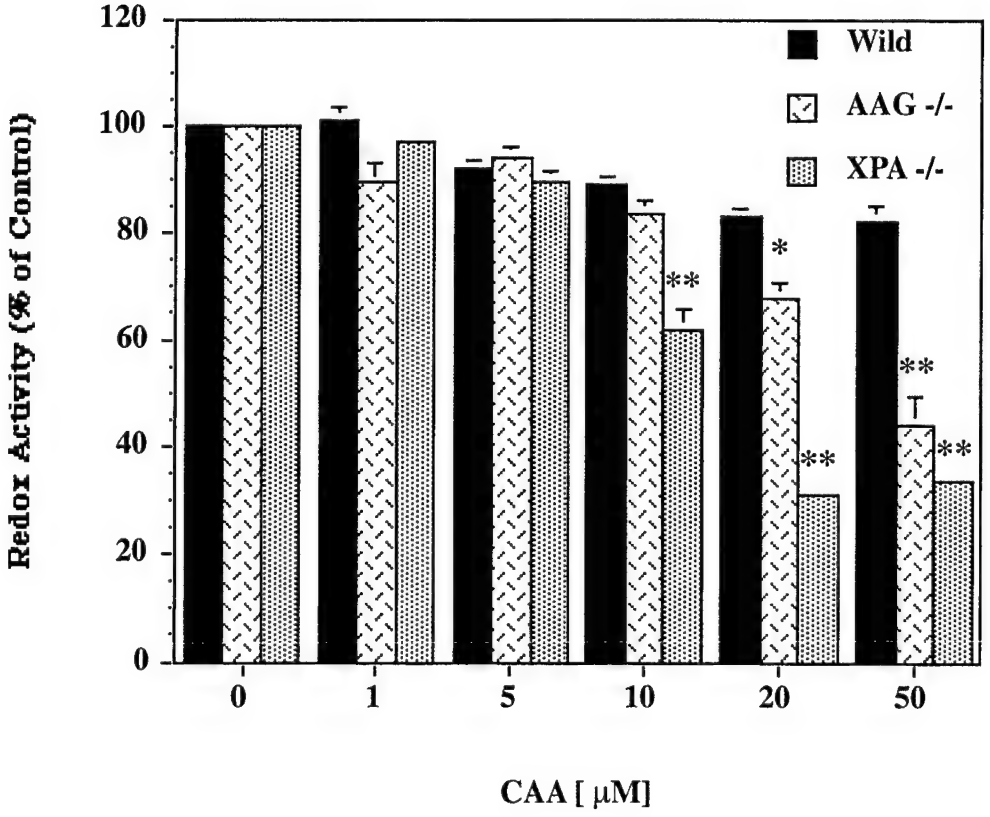
CAA [μM]


Redox Activity (% of Control)
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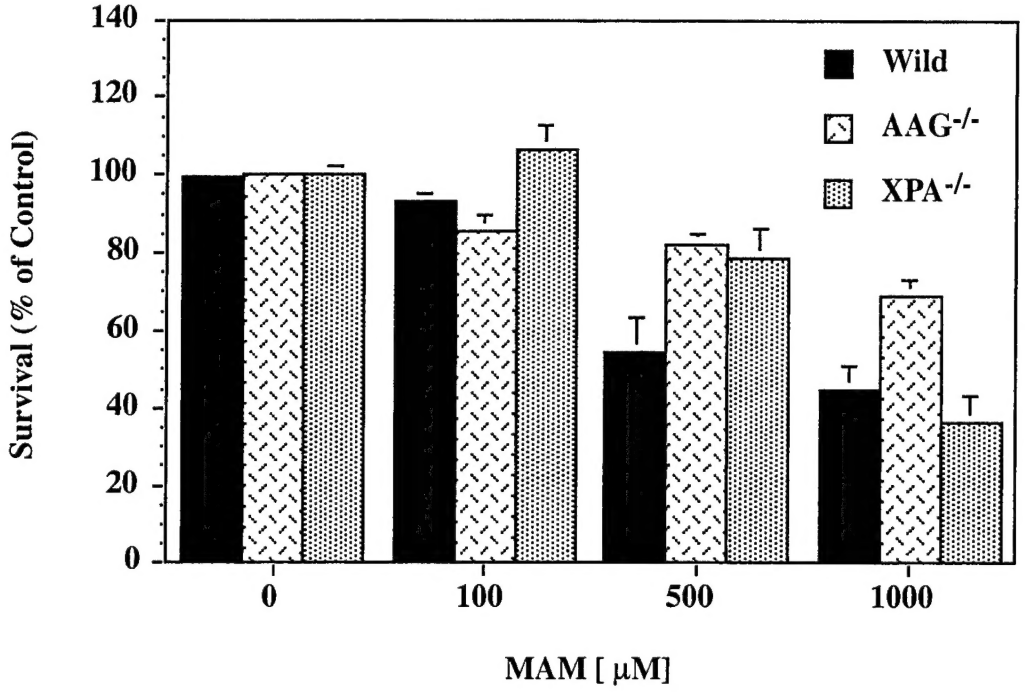
CAA [μM]

Figure 4


Survival (% of Control)
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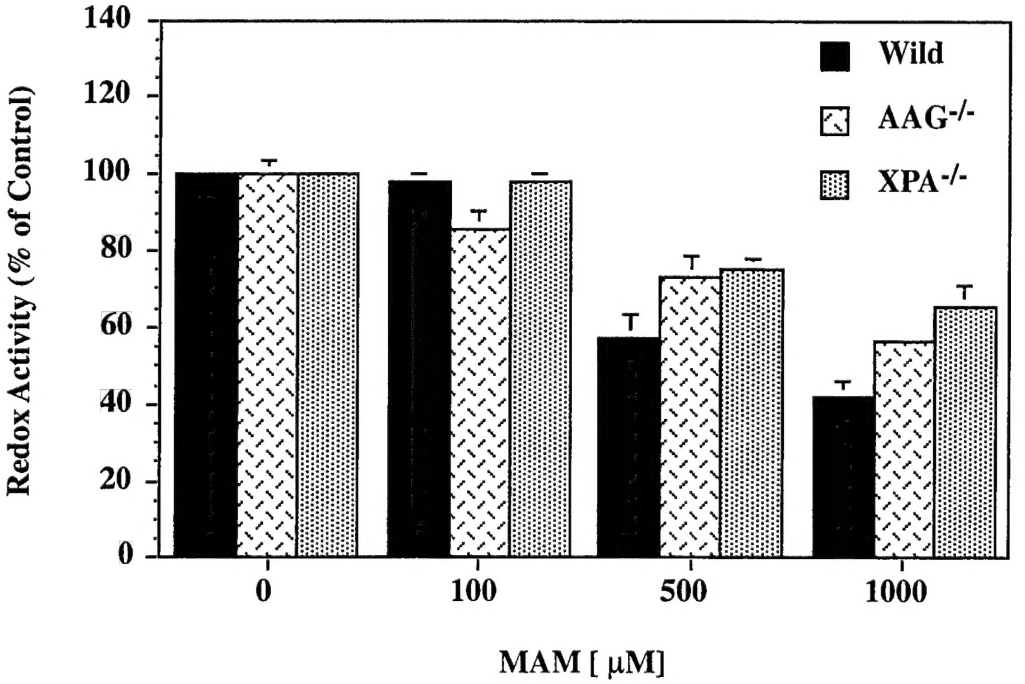
MAM [μM]


Redox Activity (% of Control)
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XPA-/-
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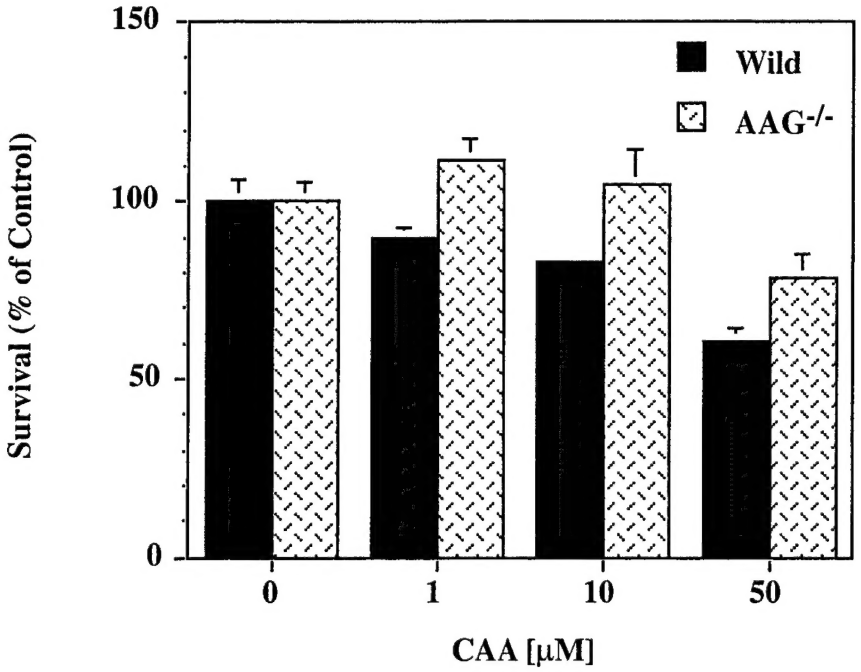
MAM [μM]

Figure 5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Survival (% of Control)
Wild
AAG-/-
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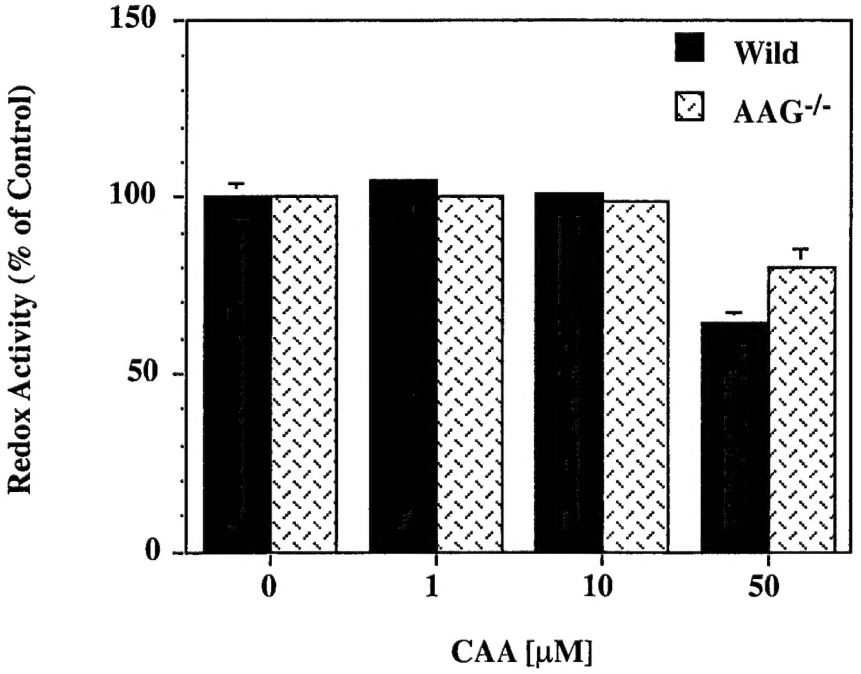
CAA [μM]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Redox Activity (% of Control)
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AAG-/-
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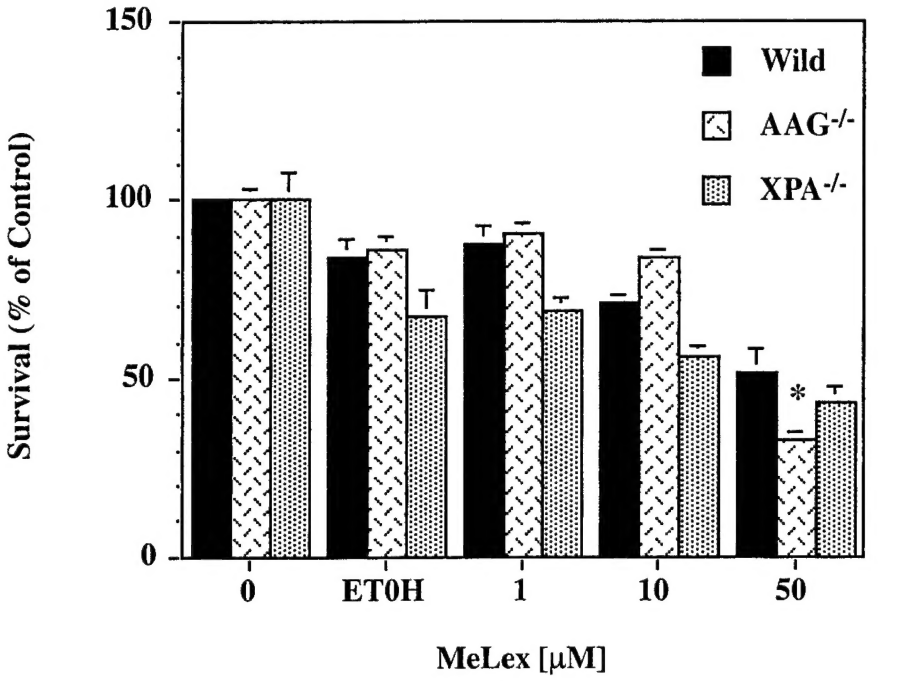
CAA [μM]

Figure 6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Survival (% of Control)
Wild
AAG-/-
XPA-/-
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0
ETOH
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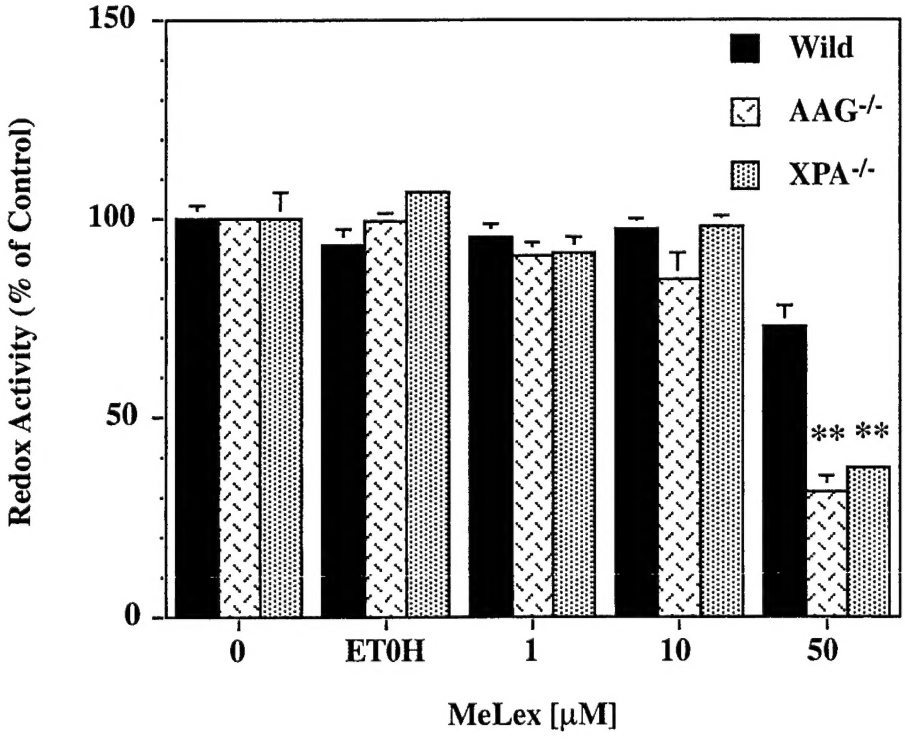
50
MeLex [μM]


150
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Redox Activity (% of Control)
Wild
AAG-/-
XPA-/-
** **
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ETOH
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MeLex [μM]

Figure 7